Isolation, Characterisation and Identification of Plant Growth Promoting Bacteria exhibiting activity against *Fusarium pseudograminearum*

A thesis submitted in fulfilment of the requirements for the degree of

Master of Science (Applied Biology & Biotechnology)

by

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DECLARATION

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Naresh Talari

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ABSTRACT

The main constraints to Australian chickpea and wheat production include several factors such as drought, biotic and abiotic stresses such as crown rot, salinity and cold; which totally contribute to losses of 10-70%. It has been found that there are practices that are helpful in controlling these stresses, such as the tolerant varieties, pesticides and crop rotation, transgenic crops and conventional breeding techniques, but these methods are not completely successful. It can be thus said that new methods need to be developed in order to minimise the biotic as well as abiotic stresses in chickpea. Plant growth promoting bacteria (PGPB) potentially represent one such novel approach and are the focus of this research. The generally perceived mechanisms of PGPB which result in reduced plant stress include competition (with a plant pathogen) for an ecological niche, secretion of inhibitory bioactive compounds, and secondary metabolic induction of systemic resistance in the plant host to a range of soil born-pathogens and abiotic stresses.

The current study focuses on the potential use of PGPB to enhance the tolerance of chickpea and wheat to crown rot caused by *Fusarium pseudograminearum.* Two strains, NM-12 and NM-33, identified as *Bacillus subtilis* and *Stenotrophomonas rhizophila* were isolated from the rhizosphere soils in Victoria, Australia (Perry Bridge). The beneficial bacterium, *Bacillus subtilis and Stenotrophomonas rhizophila* were analysed for their direct plant growth promoting effects. Direct antagonistic effect on *Fusarium pseudograminearum* was demonstrated by a dual culture assay and culture filtrate assays together with estimation of spores and fungal biomass dry weight *in vitro*.

To identify the mechanisms underlying the inhibition of the fungus by the two isolates, the bacterial exudates were assessed for the presence of a range of potential antifungal products, including lytic enzymes, hormones, antibiotics and other secondary metabolites. Strain NM-12 was shown to produce indole acetic acid (IAA) at different concentrations even at 6% salt concentration. In comparison, no IAA production was observed by strain NM-33. Further, siderophore production was moderate under control conditions but significantly increased at high salt concerntration (6%). In contrast, β- glucanase production was observed under normal as well as high salt concentrations. Interestingly, NM-12 which exhibited enhanced ability to suppress the fungal pathogen was found to possess genes encoding cyanide production and 1-Aminocyclopropane-1-carboxylate (ACC) deaminase both of which are indirectly responsible for plant growth promotion. In conclusion, the two bacterial isolates, *Bacillus* and *Stenotrophomonas* were found to be capable of promoting growth and improving the survivability of chickpea and wheat plants exposed to crown rot. These findings could be potentially extended to other crops to improve crop productivity under biotic stress.

Dedicated to my Mother

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1 CHAPTER 1

1.1 BACKGROUND AND AIMS

Fusarium crown rot (FCR) is a severe chronic cereal disease that infects the crown, basal stems and root tissues. It has recently become a common disease among cereals grown in Australia and worldwide. This is because the moist conditions at the beginning of the season enable the fungus to grow from infected stubble to an adjacent seedling (Hogg et al. 2010).

FCR is found in all the semi-arid regions that exist around the world (Chakraborty et al., 2006). It is caused by several *Fusarium sp*. Several studies reported that *F. pseudograminearum* is the common fungus that is generally related to the crown rot as observed in New South Wales and Queensland, Australia (Akinsanmi et al., 2004). All the wheat and chickpea cultivating regions in Australia are affected by this disease and it is estimated that annual losses due to crown rot are $80 million and $30-$60 million for wheat and chickpea, respectively (Verrell, 2016). Studies show that 35% of wheat crop yield loss in the Pacific Northwest of USA is due to crown rot (Smiley et al., 2005). Apart from yield associated loss, FCR infected plants may produce mycotoxins in the grains that are detrimental to human health (http://www.fao.org). It is therefore crucial to control FCR pathogen in the field.

Fungi, viruses, nematodes and bacterial are the most commonly observed causes of diseases in agricultural plants. Some species of fungi are known to cause important plant diseases and increased loss of agricultural crops. Plant pathogens need to be controlled to maintain the average level of yield both, quantitatively and qualitatively. Farmers often rely heavily on using chemical fungicides to control

these plant diseases. However, the environmental problems surrounding the widespread use of the chemicals including synthetic fungicides have led to public concern towards the use of synthetic pesticides in agriculture. Extensive use of chemical pesticides and fungicides has become a major environmental threat; for example, the use of fertilizers, pesticides and fungicides is one of the main drivers of species extinction, leading not only to a reduced global biodiversity but also to significant changes in ecosystem dynamics (Aktar et al. 2009). Despite these disadvantages, the use of agrochemicals continues to be an invaluable and powerful method to control plant disease. However, due to the negative impacts of the application of these chemicals, there is a research drive to develop sustainable, microbial-based biocontrol agents as an alternative or supplement to agrochemicals.

Biocontrol is one of the most effective alternate strategies that can be used for plant diseases (Pal et al., 2006). Biological control of plant diseases can be explained as the process in which one organism is used for impacting the activities exhibited by the other microorganisms. It is an indirect means of plant growth promotion. Biocontrol organisms may be fungi, bacteria, or nematodes. For example, a wide variety of rhizobacteria have been proven to be biocontrol agents that have been effective in suppressing a number of economically reported phytopathogens, promoting overall plant vigour and yield, either when applied to crop seeds or when incorporated inside the soil (Surgeoner 1991; Kloepper et al., 1989). Rhizospheric microorganisms appear to harbor the greatest concentration of potential biocontrol agents (Bever et al., 2012). Consequently, microbial diversity has been extensively described and also characterized, and examined for

activity so that they can behave as the biocontrol agents towards soil borne pathogens (Bever et al., 2012). Such microorganisms produce compounds which can restrict the damage to plants caused by the pathogen. These may be secondary metabolites, antibiotics or other metabolites (Bever et al., 2012).

The current project intended to conduct systematic research into the biocontrol of crown rot pathogen through the isolation of plant growth promoting bacteria from Australian soils and subsequent characterisation. Biocontrol activity against *F. pseudograminearum* was initially determined using a dual culture assay together with a culture filtrate assay followed by a number of biochemical assays and molecular analysis to allow selection of the most promising isolates. Effective suppression of FCR was the target of this project, which has resulted into the selection of isolates as bacteria that can promote plant growth.

1.2 RESEARCH FOCUS AND HYPOTHESES OF THE THESIS

The thesis investigates the growth promoting effects of plant growth promoting bacteria (PGPB) in chickpea and wheat.

Three main topics covered are:

1) ***Isolating local Plant Growth Promoting Bacteria***

   Microbial diversity and soil nutritional conditions can directly or indirectly influence the growth of plants. The microbial diversity varies with different geographical locations and local environmental conditions. Hence in this study, PGPB were isolated from three different local sites in Australia (Perry Bridge, Lardner and Rosedale).

2) Screening and characterization of isolates

Understanding the factors involved in fungal suppression by PGPB may allow identification of the antifungal compounds synthesized by bacteria. Therefore, the isolates were characterized in terms of secondary metabolite production using various biochemical assays.

3) Assessment and identification of the selected strains

Although some bacteria produce antifungal compounds that inhibit the plant pathogen, some may be harmful to humans. In addition, the reliance on morphological or biochemical based characteristics may not help in accurately identifying the bacteria. These approaches not even help in the identification of bacteria as observed at the species level. Therefore, the selected isolates were identified based on molecular biological techniques. Moreover, selected strains were characterized for the presence of secondary metabolite producing genes.

1.3 RESEARCH HYPOTHESES

The hypotheses of this project were as follows:

- Soils collected from three local sites in Australia will harbour PGPB capable of suppressing the FCR pathogen.
- The application of these PGPB bacteria would inhibit the crown rot pathogen by secondary metabolite secretion.
- The mechanisms for growth promotion among the PGPB will be identified using morphological, biochemical and molecular biology assays.

1.4 THESIS OUTLINE

Chapters 1 and 2 describe background information and review of literature, respectively. They provide context for the research and justify the research aims based on available information.

Chapter 3 presents the newly developed methodology for isolation and primary screening of plant growth promoting bacteria from Australian soils. It includes screening, selection and identification of candidate isolates.

Chapter 4 of this thesis focuses on characterization of selected bacterial strains for the production of secondary metabolites. The selected strains were assessed for their capacity to produce beneficial secondary metabolites under normal and saline conditions.

Chapter 5 contains discussions of the results that have been obtained from the study and the future opportunities that are developed based on the study.

2 CHAPTER 2: REVIEW OF LITERATURE

2.1 INTRODUCTION

Plant health is affected by several pathogenic microorganisms including bacteria and fungi that form one of the major chronic threats to the production of food and stability of the agricultural ecosystem across the globe. With the intensification of agricultural production that has been over the last few decades, agricultural producers are becoming increasingly dependent on various agrochemicals as a major, reliable option to protect and help the economic stability of the entire farming processes. This has resulted into increased use of various chemical products that lead to severe negative effects such as development of resistant pathogens that is, these pathogens are resistant to various chemical agents applied to them. Apart from this, various non-targeted environmental impacts are also caused with the excessive use of chemical compounds. Farmers and society in general have become more aware of the negative impacts of pesticides and fungicides and hence, there is a growing demand for pesticides-free food, which in turn is driving the need to develop safe, sustainable technologies as an alternative. Furthermore, the cost of the pesticides is growing day by day especially as observed in regions that are less -affluent.

Across the globe there are more and more plant diseases with fewer and fewer effective solutions. One such disease that significantly affects most crops across the globe and in particular, Australian crops such as wheat and chickpea is crown rot disease. Most commonly observed disease affecting the winter cereal crops as observed in Australia, is the crown rot disease usually caused by the fungus, *Fusarium pseudograminearum* is the most significant disease of winter cereal

(Backhouse et al., 2006). Winter cereal crops become host for the crown rot fungus resulting in significant crop losses. The most badly affected crops are barley, durum wheat, and chickpea (Backhouse et al., 2006). It has also been observed that due to excessive use of chemical pesticides, the fungus has become resistant and is causing long term impacts on the crops even when crop rotation is applied (Backhouse et al., 2006). Thus, the focus is now diverted towards biological control and it is being considered as the most effective alternative to chemical solutions to reduce the impact of harmful pathogens on crops. A large body of literature has recently become available that describes various potential uses of plant associated bacteria as potential agents to stimulate the growth of the plant as well as managing the health of the plant and soil. There are multiple species with which the plant growth promoting bacteria (PGPB) are associated with and in addition these bacteria are found to be present in the environment, particularly in the rhizosphere (Liu, 2012).

2.2 IMPORTANCE OF WHEAT AND CHICKPEA IN AUSTRALIA

Australia is a major agricultural producer and exporter, with one third of a million people employed directly or indirectly from the industry. Cereals and legumes are produced on a large scale in Australia for export and domestic consumption. With exports from Australia rising annually, wheat makes the bulk of the exports. Similarly, Australia is the largest exporter of chickpea in the world (Archak et al., 2016). Both these crops hold significant economic importance in Australia's export context. Year 2016 saw Aurizon rail transport a record amount of grains for export owing to bumper wheat and chickpea crop (Aurizon, 2016).

Wheat is considered as a staple grain for half of world's population, and represents one of the largest crops grown worldwide. One of the most important products in the agricultural sector in Australia is wheat. In Australia, production has risen from 30,000 hectares in 1900 to 4.5 million hectares by 2000 (Bell et al, 2015). The latest estimates calculate the total amount of wheat produced in Australia to be approximately 28 million tonnes, representing 15% of the total farm produce. With an export value of over $5 billion, Australia is one of the largest wheat exporters in the world (Smith, 2016). Wheat research in Australia has mainly focused on breeding disease resistant varieties suited for Australian environment.

Australia exports the second largest amount of wheat only after the United States of America. While the Australian production is only 3% of the total global produce, it still exports to 40 countries. The total export represents 15% of the total amount traded globally. Thus, the Australian wheat industry is of global significance as well of immense importance to Australia, as it employs thousands of farmers, brings foreign exchange from exports, engages significantly with internal transportation systems as well as the incalculable indirect benefits (Van Ress, 2014).

Chickpea is a pulse crop with nitrogen fixing properties. It is mostly used as a rotating crop along with cereals and canola in Australia (Rodda, 2016; Reen, 2014). Recent years have seen an increase in areas sown with chickpea. There are basically two types of chickpea grown in Australia, *Desi* and *Kabuli*. Most of the areas sown are for *Desi*. Most of the produce is exported to Asian and Middle-

East countries, with India being the largest consumer. With a bumper produce this year, Australian chickpea has seen its price surge to $1250 a tonne. According to Pulse Australia's records, Australian farmers harvested 1,013,000 tonnes of chickpea in the year 2016 (Guardian, 2016). Thus, wheat and chickpea form major exports for Australian farmers, as they bring in huge amount of foreign exchange for the country.

2.3 CROWN ROT DISEASE AND ITS PATHOGEN

Crown rot disease is caused by a soil borne fungus favoured by wet conditions. The crown or lower stem showing rotting near the soil barrier is the major symptom of the disease; many other symptoms go unnoticed and therefore untreated. The rotting may first appear on the lateral branches i.e. on one side and then start spreading to all parts of the plant Figure 2.1. The immediate symptoms that can be noticed are appearance of discoloration or dark coloured tissue at the area of infection. As the disease progresses, it makes the young foliage more susceptible to death and wilting. The leaves start to turn yellow or purple in a few cases. The other associated problems are stunted growth and darkened or tanned bark around the crown with dark sap flowing out from the diseased areas (Kamel, 2015) (Fig. 2.1).

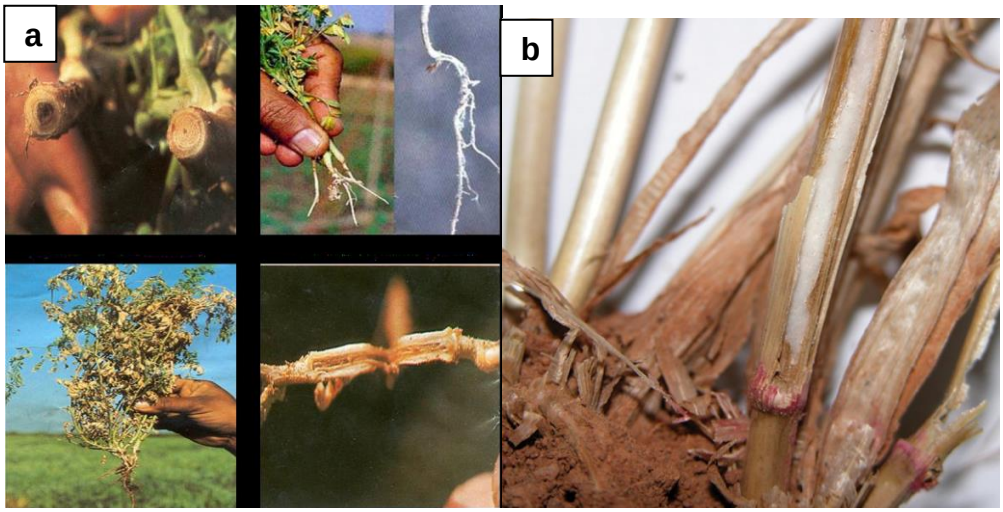


Figure 2.1: a) Symptoms of Fusarium Crown rot in Chickpea and b) Wheat (Boucher et al., 2003)

The causative agent of the disease is the fungus *Fusarium pseudograminearum*. The disease can attack winter crops such as wheat and chickpea leading to premature death and the presence of white dead heads or crowns (Li, 2017). Other *Fusarium spp*. such as *F. culmorum*, *F. graminearum* group I, *F. crookwellense*, *F. avenaceum* and *F. nivale* can also cause crown rot disease (Scott, 2004). Due to their presence within the plant stem, water movement within the plant is reduced. The *Fusarium* spp. are persistent as they readily produce spores, and survive in the soil from one season to the next from where they can reinfect crops. Thus, it is absolutely essential to manage the crops in the most efficient manner to reduce reinfection, using crop breaks and crop rotation.

In situations when the first part of the overall season yields good crop and is followed by conditions that are dry towards the end, the impacts that the crown rot has on overall yield are extremely severe. This phenomenon is said to occur because the initial season results into higher moistness in ground, making it possible for fungus to grow because of infected stubble that is present next to a

seedling. In addition to the growth of such fungus, there is a high growth of pathogen inside the plants because of moisture stress caused due to the dry conditions as observed towards the end. The damage as discussed here is said to be reduced when the entire season remains wet (Graham, 2015). As the disease spreads from the base of the plants to the stem, the plant starts showing several symptoms including:

- Grains can be pinched during harvest; this is one of the most important symptoms seen due to crown rot disease
- Formation of whitehead on the basal stem, crown around the root in particular seasons
- Pinkish fungal growth near the inner nodal areas during moist climatic conditions
- Browning of the base is also a most significant symptoms of the disease

The application of molecular techniques have allowed the development of soil DNA tests to assess the level of disease and pathogen in the soil, prior to the onset of any plant symptoms (Rowe, 2015). Regular sampling of soil especially during late summers should be carried out to identify the presence of the pathogen before the sowing of seeds. The test is said to be of particular interest and use at times when susceptible varieties of wheat are sown and when the risks generated after a non-cereal crop are being assessed.

2.4 CURRENT MANAGEMENT OF CROWN ROT

The most common management practice to control *Fusarium* crown rot is to use resistant varieties (Sandipan, 2015) and a great deal of research has been

performed using plant breeding programs to identify resistant varieties. However, despite this program, few resistant varieties have been developed. In addition to the research related to the development of resistant varieties, a number of other agronomic practices have been introduced to reduce the loss due to crown rot disease. The most important of these is probably not allowing the rotation of cereal wheat with oat, and the application of the correct amount of nitrogen to the fields, as well as allowing for proper irrigation. These changes in agricultural practices create an environment unfavourable for the pathogen and also make the host less susceptible to it. However, even the implementation of these simple management practices come with economic limitations. Therefore, the search for new resistant strains continues (Moya, 2016).

2.5 Economic Loss across the Globe and to Australia

Crown rot is seen in countries where production or cereals is very high, such as Australia, Europe and America. Despite the widespread significance of this disease to crops, there is lack of documentation of the economic loss due to the disease. However, because wheat and barley form the staple diet for more than 60% of global population, crown rot represents a major economic concern. In the USA, most of the states see losses due to crown rot every year. As much as 10% of wheat crops are damaged due to the fungus, leading to heavy economic loss (Hogg, 2010). In the Northwest of the USA, the pathogen has been reported to have destroyed 61% of wheat yield leading to a loss of $ 2 billion (Hogg, 2010). Further loss is incurred due to lowered grain quality. Similarly, again in the USA, a report suggested that there has been a reduction in wheat yield by 31% due to reduced grain quality. The loss is not limited to wheat and disease outbreak has

also led to significant economic loss of barley. Since the late 90s, several American states have lost more than 70% of the malting barley (Windels, 2000). Additionally, a number of states reported complete crop losses, and in economic terms the value exceeds $US 1 billion. Yield losses in wheat alone since 1990 have exceeded 13 Tg with economic losses estimated at $US 2.5 billion (Windels, 2000).

Other than loss due to reduced yield, crown rot disease causes more economic loss associated with mycotoxin contamination of grains. Mycotoxins are harmful chemicals having toxic effects on humans (Zhang, 2015). Due to infection with the pathogen, accumulation of the toxin occurs in the grains which can lead to serious risk to humans if levels of toxins are high. These toxins alone have huge economic impact in wheat, maize, barley and other cereal crops. Current estimations suggest that mycotoxins result into contamination of 25% of the world's crops, with an annual loss of $US 1 billion. Losses in animal productivity due to mycotoxin related health problems are an additional negative externality associated with crown rot disease. Crown rot is also a particularly concerning disease in Australia. It has done much damage to the northern wheat grain producing region of Australia. Due to the practice of growing cereal crops in close rotation, losses of wheat yields through the onset of crown rot disease have reached 100% in some areas. It is estimated to cost Australian growers $US 97 million annually (Matny, 2015). The average yield loss due to crown rot disease in Australia is 25% in wheat, 20% in barley and 58% in durum. It has been calculated that during heavy disease occurrences, the yield of wheat in Queensland was reduced by 50% (Matny, 2015).

2.6 PLANT GROWTH PROMOTING BACTERIA (PGPB)

The soil is filled with various microorganisms including bacteria, algae, fungi and protozoa. Of these, bacteria are the most abundant, reportedly up to 10^8 colony forming units per gram of soil. However it is well known that using only one isolation media can result in the growth of less than 1% of the bacterial population (Kathryn, 2005). The number and type of bacteria in a soil depends on the environmental condition of that area; in a soil where there is excessive environmental stress, the number of culturable bacteria found will generally be lower (Pham, 2012). Environmental stress factors include temperature, moisture, and the presence of chemicals and salts. The number and diversity of microbes present is also dependent on the crops growing in the soil (Koorem, 2014). The distribution of bacteria in soil is not homogeneous; some areas of soil have a higher concentration of micro-organisms compared to other areas. For example, greater numbers of bacteria are found around the roots of some plants compared to the bulk soil. This is because of the presence of high concentration of sugars, amino acids and other important organic acids and molecules that are secreted (exuded) by the plant roots.

The soil contains both beneficial and deleterious (e.g. plant pathogens) bacteria that greatly affect the growth of crops. Pathogenic bacteria may infect the plant resulting in reduced growth and yield and perhaps plant death. On the contrary, beneficial bacteria help the crops to grow, to become stress resistant and to be able to solubilize essential minerals (McKersie, 2013). However, changes in environmental conditions can result into changes into the way a plant is influenced by a given bacterium. For instance, a particular bacterium that helps a plant to

grow by fixing nitrogen or solubilizing phosphorus may not be helpful to the plant when the soil is heavily loaded with chemical fertilizers. In addition to this, a particular bacterium may affect plants differently. For example an IAA-producing mutant bacterium was shown to stimulate root development in blackcurrant cuttings whereas the bacteria inhibited root development in cherry cuttings. It is likely that the blackcurrant cuttings contain suboptimal level of IAA which was further enhanced by the mutant bacteria whereas in cherry cuttings the level of IAA was optimal and addition of the mutant led to an inhibitory effect (Bellini, 2014).

Generally, plant growth promoting bacteria (PGPB) are free living and tend to establish pecific symbiotic relationships with the plants (Glick, 2014). The bacterial endophytes colonize in specific regions of the plant and help in promoting plant growth. The bacteria can either directly or indirectly promote plant growth by facilitating the acquisition of resources or modulating plant hormone levels, or have direct or indirect stimulatory effects on the growth of the plant (Fig. 2.2). The *Rhizobia* spp. represents one of the most widely studied PGPB from various physiological, molecular biological and biochemical perspectives. Examples of other bacteria that have been found to enhance plant growth include *Enterobacter, Arthrobacter* and *Pseudomonas*. However, not all PGPB have nitrogen fixation as their mode of action; there are various mechanisms that are used by these bacteria to help the plants (Glick, 2012). These approaches can be direct or indirect mechanisms (Figure 2.2).

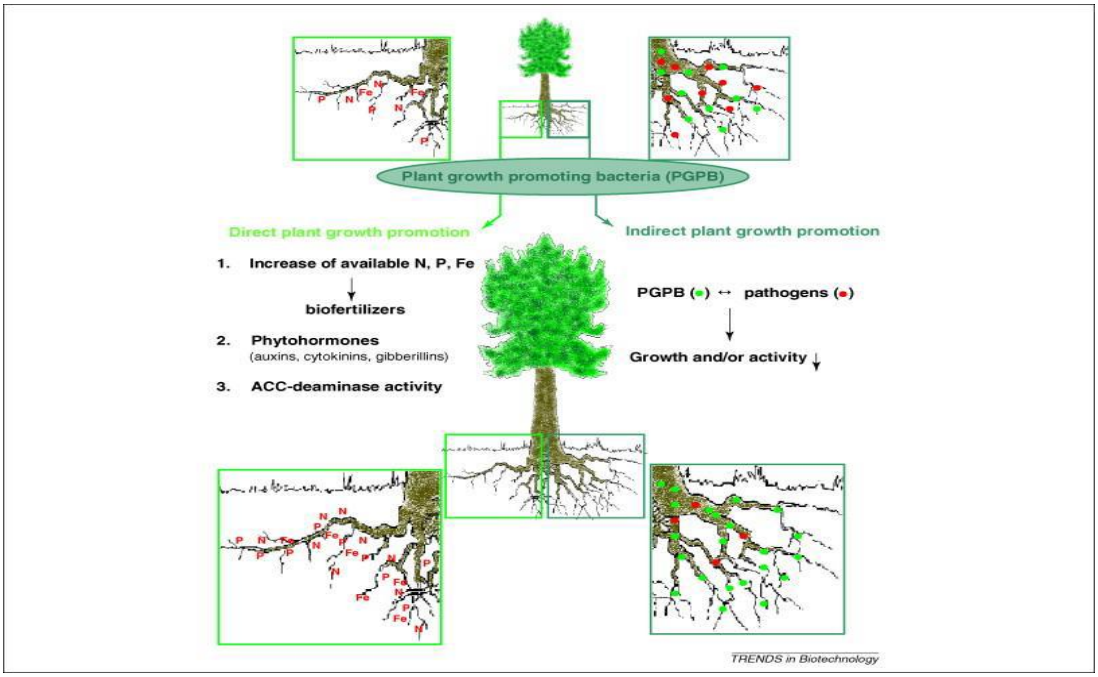


Figure 2.2: Overview of the mechanisms of biocontrol (Beauregard et al., 2013)

2.7 DIRECT MECHANISMS

Facilitating acquisition of resources: Not all agricultural soils contain sufficient amount of compounds that will support optimal or even sub-optimal growth of the plant. To address this problem, farmers across the globe are now highly dependent on chemical supplements; however, this has several negative impacts such as depletion of non-renewable resources, hazards for humans and excessive cost to the farmers. One of the most widely studied mechanisms of how bacteria promote plant growth includes the process of how they are able to provide nutrients and other resources to the plant such as fixation of nitrogen, phosphorus and iron. Therefore, researchers have been studying rhizobacteria and mycorrhiza that are capable of fixing nitrogen or solubilizing phosphorus, respectively. In addition to the Rhizobacteria, there are several other free living bacterial species

that are also able to fix nitrogen for the plants although they are known to fix very small amounts of the nitrogen that is required by the plant (Bernhard, 2012).

2.7.1 Phosphate Solubilisation

Phosphorus in the soil is present either in an organic form such as inositol phosphate or an inorganic form such as apatite. Despite the fact that phosphorus is present abundantly in the soil, the phosphate is generally in an insoluble form and hence, not available for the plants. Chemically added phosphates in the form of fertilizers are often immobilized immediately upon application and become unavailable to the plants. Therefore, even in soils amended with fertilizers, there is always very limited bioavailability of phosphorus. This makes it one of the most essential compounds for agricultural production. Therefore, bacteria which can solubilise and mineralize the phosphorus in the soil are of great importance. Inorganic phosphorus is solubilised as a result of the action of low molecular weight organic acids which are synthesized by the soil bacteria. Mineralization of organic phosphorus takes place through the action of bacterial phosphatases that catalyze the hydrolysis of phosphoric esters (Kumar, 2012).

2.7.2 Iron Sequestration

Though iron has been found to be the fourth most abundant element as present on earth; it is not readily available in aerobic soils. This is because the ferric ions which are sparingly soluble predominate; hence living organisms assimilate extremely low amounts of iron. Both, bacteria and plants require high levels of iron. This competition creates problems in the rhizosphere where bacteria, plant and fungi are all seeking iron. In order to optimise iron sequestration, bacteria synthesize siderophores that have a high affinity for ferric ions. There are more

than 500 known siderophores. Researchers have shown that when mung bean plants were inoculated with siderophores producing bacteria, *Pseudomonas* spp., an enhanced level of chlorophyll was observed (Alexander and Zuberer, 1993). When the plants are subjected to metal pollution or any other form of environmental stress, the use of soil bacteria for providing iron to the plants becomes even more crucial (Rungin, 2012). Similarly, the Fe-pyroverdine complex synthesised by *Pseudomonas fluorescens* was found to be taken by *Arabidopsis thaliana* resulting in improved plant growth. Plant iron concentrations also affect the structure of the bacterial community found in the rhizosphere; for example, a transgenic tobacco plant that overexpresses the ferritin gene was shown to accumulate high levels of iron, making iron less available in the rhizosphere and as a result a significantly different bacterial community was found in the rhizosphere compared to non-transgenic tobacco plants (Mendes, 2013).

2.7.3 Modulating the levels of phytohormones

Plant hormones play an important role in its growth and development and in response environmental stressors. A plant has to face multiple stresses that severely affect its growth until either the plant adjusts its metabolism or the stress is removed. When growth limiting environmental stress is encountered by a plant, it attempts first to adjust its endogenous phytohormones so that it can minimize the effect of environmental stress. This strategy proves to be successful sometimes; however often the microorganisms present in rhizosphere also produce or modulate the phytohormones that affect the hormonal balance of the plants and their responses to stress (Vacheron, 2013).

2.7.3.1 Expression and modulation of cytokinins and gibberellins

It has been found that in general, many soil bacteria, particularly PGPB, can produce cytokinins or gibberellins or even both. For example, studies have shown the presence of cytokinins in the cell-free medium of *Azotobacter*, *Pantoea*, *Rhodospirillum*, *Rhizobium, Bacillus subtilis*, *Pseudomonas fluorescens* and *Paenibacillus polymyxa* (Pérez-Montaño, 2014). There are several reports regarding the production of these two hormones by PGPB. However the detailed role of how these bacteria synthesize these hormones is yet to be studied. There are some strains of phytopathogens that are also capable of synthesizing cytokinins. In contrast, PGPB produce relatively low levels of cytokinins compared to those produced by the phytopathogens (Santoyo, 2012).

2.7.3.2 Indoleacetic Acid (IAA)

Indoleacetic Acid is the most common and widely studied auxin among all other naturally occurring auxins and often in literature the terms ‘auxin’ and ‘IAA’ are used interchangeably. IAA participates in cell division, differentiation, extension and stimulation of the seeds, germination of tuber, increasing the rate of development of root and xylem, controlling the vegetative growth process, initiating the formation of adventitious and lateral roots, mediating various responses to light and gravity, primarily affecting photosynthesis, pigment formation, biosynthesis of different metabolites and resistance to stress (Ljung, 2013). IAA therefore plays a role in almost every process associated with plant growth. The responses of a plant to IAA may vary from plant to plant; some plants may be sensitive to IAA but others may not be. This may be the same when specific parts of the plant are involved. For example, the development of roots

may be sensitive to IAA but not shoot development. Acquisition of IAA that is secreted because of the bacteria as present in soil, can result into alterations of endogenous pool of plant IAA. In this case, the IAA synthesized by the plant determines whether the IAA secreted by the bacteria will suppress or stimulate the plant growth. The endogenous IAA in the plant roots may be present at optimal or suboptimal level and the addition of IAA from the bacteria may alter the level of IAA to either supraoptimal or optimal levels that result in inhibition or promotion of growth, respectively. The role of IAA synthesized by the plant growth promoting bacteria, *Pseudomonas putida* GR12-2, in root development of canola was reported. Inoculation of seed with wild type *P. putida* GR12-2 induces 30-50% longer roots compared to the seed treated with the IAA-deficient mutant (Patten and Glick, 2002). In contrast, inoculating mung bean with a mutant from the same strain that overproduces IAA resulted in the formation of greater number of shorter roots as compared to the control (Patten and Glick, 2002). These results indicate the combined effect of auxin on promotion of growth and ethylene inhibiting root elongation. The bacterial synthesized IAA incorporated by the plant stimulated the activity of ACC synthase, an enzyme that increases the ethylene concentration resulting in inhibition of root elongation (Lugtenberg, 2013). Overall it has been found that bacterial IAA increases the surface area and length of roots, thus providing greater access to the nutrients present in soil. Apart from this, the bacterial IAA production results in a reduction in the thickness of the plant cell wall, facilitating increased root exudation and providing additional nutritional support to the growth of bacteria in rhizosphere. Most of the *Rhizobium* strains that have been studied so far have been found to produce IAA. Several studies

suggest that an increase in the level of auxin in the host plant is important for the formation of nodule. Thus, mutant bacteria that are not capable of producing enough IAA induce very few nodules compared to the wild-type strain. Additionally, the nodules induced by the mutants that produces low IAA (Glick, 2012).

2.8 INDIRECT MECHANISMS

The potential of biocontrol bacteria for the purpose of supporting overall growth of plants is widely studied by the scientific community for two reasons: firstly, it helps in understanding the underlying mechanisms through which biocontrol bacteria act and secondly, it allows their potential use to replace the chemical pesticides that are environmentally damaging. Both these objectives complement each other since once the mechanism is known, the use of biocontrol bacteria can be explored and trials can be performed.

2.8.1 Production of Antibiotics and Lytic Enzymes

The production of antibiotics and lytic enzymes by PGPB helps in inhibition of plant pathogens, especially fungi. A number of antibiotics have been isolated and studied in detail resulting in the commercialisation of biocontrol bacteria. However, the regular application of biocontrol agents that produce only one type of antibiotic may lead to resistance among plant pathogens. To address this issue, researchers are focusing on identification of organisms capable of producing more than one antibiotic and also hydrogen cyanide. Although hydrogen cyanide itself doesn't have any biocontrol activity, it has been shown to act synergistically with antibiotics for the inhibition of plant pathogens.

A number of enzymes are also produced by these biocontrol bacteria. Among these enzymes are chitinases, proteases, β-1, 3 glucanases and lipases. All these enzymes have the capacity to lyse the fungal cell wall. These enzymes have biocontrol activity against a range of plant pathogenic fungi. The plant pathogens that have high susceptibility to these enzymes include *Botrytis cinerea*, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Phytophthora spp*., *Pythium ultimum* and *Rhizoctonia* (Glick, 2012)

The following represent a list of bacteria commonly used as biocontrol agents:

- *Gliocladium catenulatum* (used in the suppression of *Pythium, Phytophthora, Rhizoctonia,* and *Botrytis*)
- *Streptomyces griseoviridis* (used in the suppression of *Fusarium, Pythium,* and *Phytophthora*).
- *Streptomyces lydicus* (used in the suppression of powdery mildew)
- *Beauveria bassiana* (used to control whitefly, aphids, and thrips)
- *Trichoderma harzianum* (used in the suppression of *Rhizoctonia, Pythium*)

2.8.2 Production of Siderophores

A number of PGPB do not produce any biocontrol agents yet they are capable of showing biocontrol activities through the siderophores they produce. Siderophores prevent plant pathogens from accumulating iron thus limiting their potential to grow and proliferate. This mechanism is thought to represent a promising biocontrol

approach as the iron accumulating capacity of PGPB is greater than that of the fungal pathogens. If the fungal pathogens lack iron, they are unable to proliferate in the rhizosphere region of the plant. Thus, these PGPB effectively eliminate fungal pathogens through competitive inhibition by preventing their growth. In this instance plant growth is unaffected by the siderophores as the plants can grow at much lower concentration than that required by the plant pathogens. Additionally, plants can take up the PGPB iron-siderophore complex following binding (Joseph, 2012).

Siderophore production by PGPB has been confirmed to assist in preventing the growth of the plant fungal pathogen (Saraf, 2014). This was demonstrated in studies in which strains were mutated so that they were unable to produce siderophores. These mutant strains subsequently showed reduced inhibitory activity compared to the wild type variety. Another study reported that strains which overproduced siderophores exhibited more activity against fungal strains compared to the wild type strains (Santoyo, 2012).

3 CHAPTER 3: ISOLATION, SCREENING, SELECTION AND IDENTIFICATION OF PLANT GROWTH PROMOTING BACTERIA

3.1 INTRODUCTION

Fusarium pseudograminearum is the most common causative pathogen of fusarium crown rot (FCR) in chickpea and wheat in Queensland and New South Wales, Australia (Akinsanmi et al., 2004). All wheat and chickpea growing regions in Australia were infected by this disease in 2015. Annual financial loss owing to FCR was estimated to be $80 million and $30-60 million Australian dollars in wheat and chickpea, respectively (Murray and Brennan 2009; Verrell 2016). The study focuses only on chickpea and wheat, as both are major winter pulse crops grown in Australia (Victoria, Western Australia, South Australia, New South Wales and Queensland). Further, Australia is the largest exporter of the chickpea and wheat.

In addition to Australia, there are several other cereal growing regions that have shown higher growth of FCR. This might be attributed to the high intensity of cropping together with wider usage of minimum tillage for moisture conservation (Hogg et al. 2010). In fact, grains can also exhibit mycotoxins because of plants that are infected by FCR (Mudge et al. 2006) and hence existence of any of such toxic compounds in different varieties of feeds as well as food is said to be a major safety concern.

Biocontrol is an effective strategy that might be used for reducing FCR and suppressing its causative pathogen. Multiple fundamental solutions as well as

biology for resolution of agricultural practical problems have been determined by research done in the field of biocontrol (Hagen & Franz. 1973). Biological control of plant diseases is hence said to be the process in which the activities of plant pathogen get affected by the organisms. It is an indirect means of plant growth promotion (DeBach, 1974).

Biocontrol for promoting plant growth and reducing the impact of FCR might be introduced with the help of 'plant growth promoting bacteria' (PGPB). All bacteria that are beneficial for plant development are called PGPB (Klopper 1986). These bacteria potentially improve plant growth by increasing the nutrient uptake and inhibiting pathogens. They can therefore be used in agriculture to reduce the utilisation of chemical pesticides and fungicides (Diaz-Zorita and Fernandez Canigia 2009). Several studies reported that *Pseudomonas, Azospirillum, Azotobacter, Bacillus, Xanthomonas, Klebsiella, Enterobacter* and *Serratia* may promote plant growth (Doutt, 1964).

In accordance with literature, a number of PGPB could be used as effective biocontrol agents as they act by suppressing a variety of economically important phytopathogens and often promote overall plant vigour and yield (Turner and Beckman 1991). Most of the studies have described higher diversity of rhizospheric microorganisms; in fact there have been tests for activities such as the use of biocontrol agents against soil borne pathogens in multiple cases. Such PGPB is hence said to result into generation of different produce substances that can restrict the overall damage caused due to phytopathogens, e.g. by producing secondary metabolites.

Usage of PGPB for sustainable agriculture has increased over the last couple of decades. Ruppel (1987) reported that the inoculation of PGPB significantly improved plant growth and yield. Biological N_2 fixation provides a ready supply of nitrogen for flora as part of environmentally friendly agricultural practices. Apart from fixing N_2, PGPB can have a positive effect on plant growth in various other ways. These include enhancing nutrient uptake through the synthesis of phytohormones and nutrients, enhancing stress resistance, inhibiting plant ethylene synthesis, mineralising natural phosphate and also through solubilising inorganic phosphate (Lucy et al., 2004). Additionally, PGPB can influence plant growth by enhancing the germination rate, protein content, chlorophyll content, nitrogen content, tolerance to drought and salinity and cold, root weight and shoot weight (Dobbelaere et al., 2003). PGPB also can promote plant growth by siderophore production that enhances iron uptake in cases of iron shortage in rhizosphere.

Alternately, in alkaline soils, siderophore producing bacteria can reduce iron availability to suppress the growth of *Fusarium sp*. (Winkelman 2002). Studies also show that the growth-enhancing potential of a few microorganisms can be exceptionally unique to certain plant species (Sala et al. 2007). The objective of this experiment was to screen bacteria from Australian soils and identify PGPB isolates that can suppress *F. pseudograminearum*, the causal agent of crown rot in chickpea and wheat.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and Raw materials

Chemicals and raw materials used in this study were all obtained from Sigma Aldrich, Australia.

3.2.2 Pathogen

Fusarium pseudograminearum strain was obtained from Microbial Life Laboratory collection, RMIT University, Bundoora.

3.2.3 Soil samples

Soil samples for isolation of PGPB were collected with the help of our industrial collaborator, Ternes Consulting Ltd. and obtained from upper layer of rhizosphere (5-10 cm deep) where the bacterial population is high. Approximately 100 g soil was collected per site using sterile polythene bag and a sterile trowel. Soil samples were collected from three different sites in Victoria, Australia (Perry Bridge, Lardner and Rosedale). Soil samples were returned to the laboratory within 24 h of sampling and stored at -80^{o} C prior to use.

3.2.4 Preparation of different media to obtain maximum recovery of PGPB during isolation

Several selective media were used in order to maximise the recovery of bacteria from soil during the isolation process. The media selected and their compositions are presented below (Table 3.1 to 3.3).

Table 3.1: Composition of Soil extract agar

Soil extract agar (SEA)	
Ingredients	**g / Litre**
Glucose	1
Dipotassium phosphate	0.5
Soil extract *	17.75
Agar	15
Final pH (at 25°C)	6.8 ± 0.2
*Soil extract was prepared by mixing 5 g of soil in 1L H_2O, filtered and then autoclaved.	

Table 3.2: Composition of Tryptone soy agar

Tryptone soy agar (TSA)	
Ingredients	**g / Litre**
Tryptone (pancreatic digest of casein)	15
Soytone (papaic digest of soybean meal)	5
Sodium chloride	5
Agar	15
Final pH (at 25°C)	7.3 ± 0.2

Table 3.3: Composition of Nutrient agar

Nutrient Agar (NA)		
	Ingredients	***g / Litre***
1.	*Peptic digest of animal tissue*	*5*
2.	*Sodium chloride*	*5*
3.	*Beef extract*	*1.5*
4.	*Yeast extract*	*1.5*
5.	*Agar*	*15*
6.	*Final pH (at 25°C)*	*7.5*

All media were sterilized in an autoclave for 15 minutes at 121°C. The media were then allowed to cool, dispensed in Petri dishes, and allowed to solidify.

3.2.5 Rapid isolation and *in vitro* screening of effective bacteria

To isolate bacteria, an initial dilution of the soil (1 g) was made in sterile distilled water (10 mL). The contents were mixed to suspend all organisms from the soil into the broth and the tube was allowed to sit at 25°C (room temperature) for a time period of around 30 minutes such that majority of the soil particles could settle. Seven fold serial dilutions of the soil microbial suspension were made with phosphate saline water. Finally, the upper liquid broth was streaked on primary streak plates where fungal spores were inoculated previously.

The innovation in this methodology is the prior inoculation of agar with *F. pseudograminearum* spore suspension. The fungal spore suspension was obtained by pouring 15 mL of sterile water onto a freshly subcultured fungal plate and incubating for 15 min. The mycelia were scraped using a sterile spatula and

filtered into sterile Falcon tubes using sterile cheese cloths. Spores (50 µL of 10^4 cfu/mL) were inoculated and spread evenly all over the agar plate and allowed to dry for 2 minutes. Subsequently, 50 µL of soil bacteria (10^4 cfu/mL) were inoculated and spread with the help of sterile spreaders. Therefore, equal concentration (10^4 cfu/mL) and volume (50 µL) of the bacterial and fungal cultures were plated on various isolation media. The plates were labelled and incubated at 28^{o}C for 48 hours. Small, clear zones of fungal inhibition were observed around some bacterial colonies. Single bacterial colonies were picked from the clear zones with a sterile tooth pick and streaked on freshly made agar plates until pure cultures were established.

3.2.6 Selection of antagonistic bacteria by dual culture assay

The isolates were tested for their antagonistic activity *in vitro* against *F. pseudograminearum* using a dual culture method. A 1 cm fungal disc from a five day old mycelial mat was inoculated in the centre of a potato dextrose agar (PDA) containing Petri dish. The PDA facilitates fungal as well as bacterial growth (Jin & Ding, 2011). The new bacterial culture was single streaked at a distance of approximately 3 cm from the fungal disc. The plates were incubated at 28^{o}C and the growth was observed for 48 hours.

The bacterial isolates were divided into three types depending upon their antifungal activity. Type one included all those isolates which displayed significant antagonism against the fungus. The isolate which displayed partial antagonism against the fungus were type two whilst the ones with no antifungal activity were type three. The radial growth of fungus in treatment and control plates was

measured and the antifungal activity was calculated as a percentage using the following formula:

$R = R_2 / R_1 * 100$

R_1- radial growth (control)

R_2 - radial growth (treatment)

The type one isolates were evaluated further.

3.2.7 Identification of selected bacteria

Pure cultures of selected bacteria were obtained by streaking on nutrient agar. A single colony of selected bacteria was used to inoculate nutrient agar and grown overnight with shaking (100 rpm at 28^{o}C). Bacterial genomic DNA was extracted from cells of overnight cultures using the MoBio DNA mini extraction kit (Qiagen, CA) according to the manufacturer's instructions.

For phylogenetic analysis of selected bacteria, partial sequencing of the 16S rRNA gene was undertaken. The 63F and 1389R primers (Table 4) were used to amplify the 16S rRNA gene by PCR. The 50 µL PCR reaction included 1X PCR Supermix (20 mM Tris-HCl, 5 mM KCl, 1.5 mM, 200 µM of each dNTP, 1 U *Taq* DNA polymerase; Invitrogen, California), 0.5 µM of each primer, 1 µL of diluted genomic DNA (10 ng) and an additional 0.5 mM $MgCl_2$ to produce a final concentration of 2 mM $MgCl_2$. Cycling conditions included an initial denaturation step at 94^{o}C for 4 min; 25 cycles of 94^{o}C for 30 sec, 55^{o}C for 30 sec, 72^{o}C for 90 sec; and a final extension at 72^{o}C for 10 min. The PCR products were tested on a 1.5% agarose gel stained with SYBR safe (Thermo Fisher Scientific, Australia).

Table 3.4: Primers used for PCR amplification of bacterial and fungal genes

Primer name	Microbial target	Sequence (5' to 3')	Reference
63F	Bacteria	CAGGCCTAACACATGCAAGTC	(Osborn et al., 2000)
1389R	Bacteria	ACGGGCGGTGTGTACAAG	

The PCR products were purified and sequenced at the Australian Genome Research Facility (AGRF), Melbourne, Australia. The Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information (http://www.ncbi.nml.nih.gov/BLAST) was used to compare of DNA sequence to those in Gene Bank.

3.2.8 Culture filtrate assay

Pure cultures of the type one bacterial isolates that displayed high antagonistic activity in the dual culture assay were inoculated in nutrient broth and incubated on a rotary shaker (180 rpm) at 28^{o}C for 72 h. The pH of the medium was adjusted to 7.0 before sterilisation. At the end of incubation period, the bacterial cultures were centrifuged at 5,000 rpm for 10 min. The superannuant was filtered through 0.22 µm membrane filter under aseptic conditions and the filtrates were inoculated onto sterilised PDA plates (Table 5) at a 20% (v/v) concentration: 20 ml of the inoculated agar were poured into each Petri plates and allowed to solidify. A fungal mycelial disc of 5 mm was inoculated in the centre of each plate as above. Non-inoculated PDA plates were used as the control. The radial growth of the fungus was measured by comparing treatment and control plates for seven days.

Table 3.5: Composition of Potato Dextrose agar (PDA)

Potato Dextrose agar (PDA)	
Ingredients	**g / Litre**
Potato dextrose broth	24
Agar	2
Deionised water	up to 1000 mL

3.2.9 Antifungal activity in broth

To study the antifungal activity of the type one bacteria in broth, viable cells of the bacteria were inoculated into autoclaved Glucose Yeast extract broth (GY broth) (Table 3.6) and incubated at normal conditions. The bacteria should theoretically increase in number by utilising the nutrients in the media and reach a maximum count during the exponential phase before stopping cell division and entering the stationary phase. The sporulation of fungi in liquid culture is still debated; however spores increase the fungal mass by producing a mycelial sheath.

If the bacteria and fungi are inoculated into the same media, they must compete for the nutrients and space to grow. They may therefore produce secondary metabolites to suppress each other's growth. If the bacteria have the upper hand, fungal growth would be supressed or *vice versa*.

Equal concentration and volume (20 µL of 10^4 cfu/mL) of selected type one bacterial strains (NM-12, NM-33 and a mixture of both cultures) and the fungal spores were inoculated into the GY broth. The cultures were incubated at 28^{o}C for

7 days. Broth inoculated with only the bacteria or fungus served as controls. In order to investigate which bacterial isolate had the strongest antifungal activity, the bacterial and fungal cells were counted every 4 hours until the stationary phase of bacteria in control conditions (24 h). A haemocytometer was used for cell counts.

Table 3.6: Composition of Glucose Yeast (GY) extract

Glucose Yeast extract	
Ingredients	g / Litre
Yeast extract	5
Glucose	2
Monopotassium phosphate	0.5
Dipotassium phosphate	0.5
Magnesium sulphate	0.3
Sodium chloride	0.01
Manganese sulphate	0.01
Zinc sulphate	0.0016
Copper sulphate	0.0016

Cobalt sulphate	0.0016
pH	6.8 ± 0.2

3.2.10 Antagonistic activity by fungal biomass dry weight

The two selected isolates (NM-12 and NM-33) and the combination of their cultures (consortium) were tested for their antagonistic activity *in vitro* against *F. pseudograminearum* by determining the effect of bacterial strains on the fungal dry weight. Equal concentrations (20 µL of 10^4 cfu/mL) of the bacterial and fungal spores were inoculated in the GY broth. For the consortium, 10 µL of each strain containing 10^4 cfu/mL were used. They were incubated for 8 days at 25^{o}C to attain the maximum growth, and thereafter the mycelium was separated from bacteria and media by vacuum filtration and the dry mass was estimated after heating in an oven at 90 ^{0}C until a constant weight was measured. Each measurement was performed in triplicate. The dry weight (mg) of fungus was calculated according to the formula below (Singh et al., 2012):

$I = T – C \times 1000$

Where,

I = dry weight of fungi (g/ml)

C = dry weight of empty filter paper

T = dry weight of the fungal component and filter paper

3.3 RESULTS AND DISCUSSION

3.3.1.1 Isolation of bacteria from Australian agricultural soils

Biological control can be used as an effective method for the overall management of diseases as well as pests in the field of agriculture and is better than the conventional method of using chemical pesticides, which often pose significant threat to the environment including being harmful to the beneficial organisms present in the soil. Currently, the development of new eco-friendly methodologies are based on the identification of new chemical as well as biological sources that can be effective for pest management without damaging the environment. Even though the use of different microorganisms as control agents has been established, the growth of soil borne pathogens can be suppressed via the antibiotics that are generated by bacteria isolated from soil. In the current work, the bacteria from Australian soils were isolated with the intention of using these isolates as biological agents in the pest management of crown rot caused by *F. pseudograminearum.*

Strains which produced tiny zone of clearance (as represented in Figure 3.1A, 3.1B) were picked and pure cultures were made by sub streaking. Based on the morphological observations, such as colony formation on top of the medium, cell shape, type of colony and reduced activity on the fungal pathogen, 13 out of the 45 isolates were not considered for further analysis. They were thought to be sibling isolates belonging to the same species.

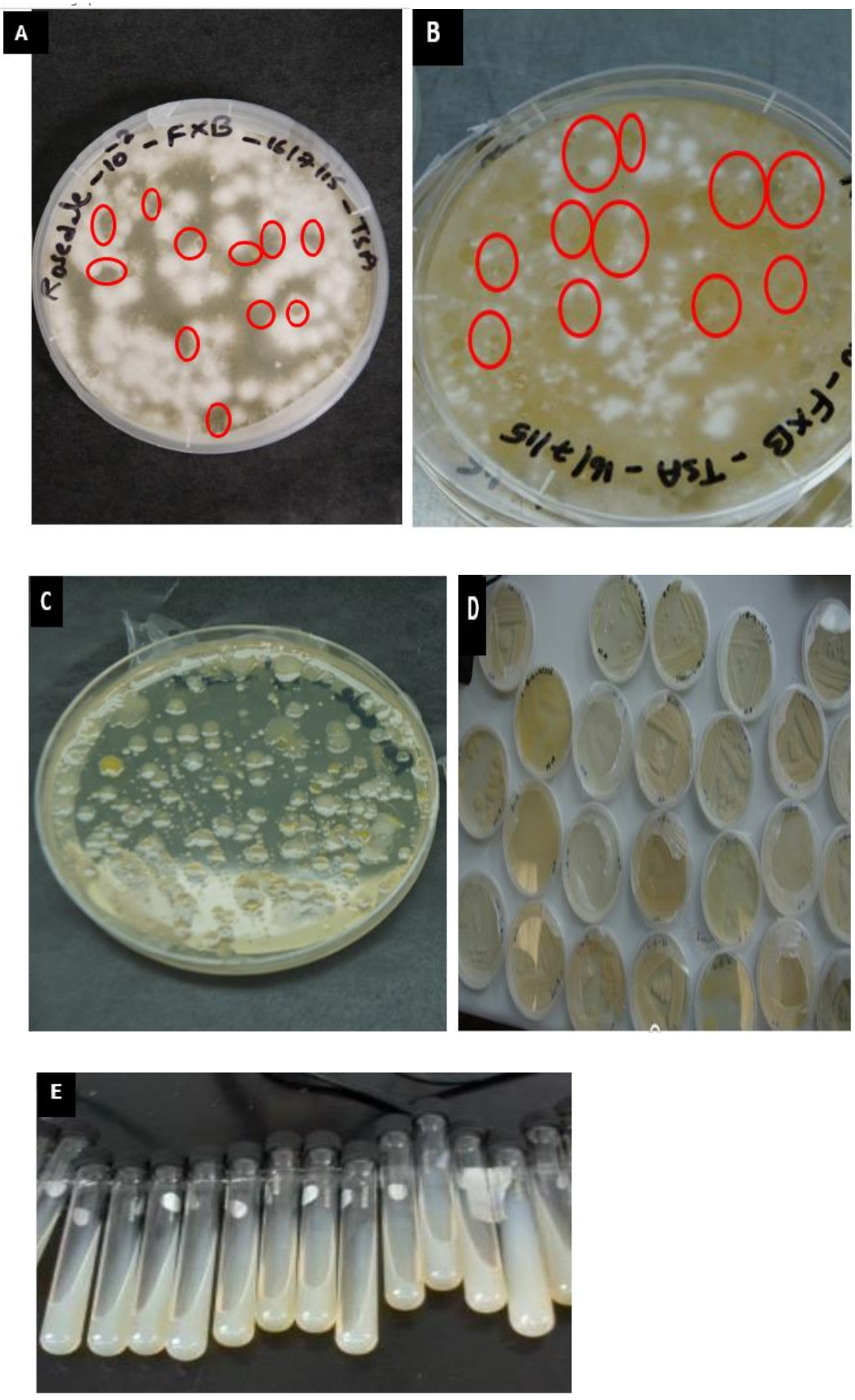

A
B
C
D
E

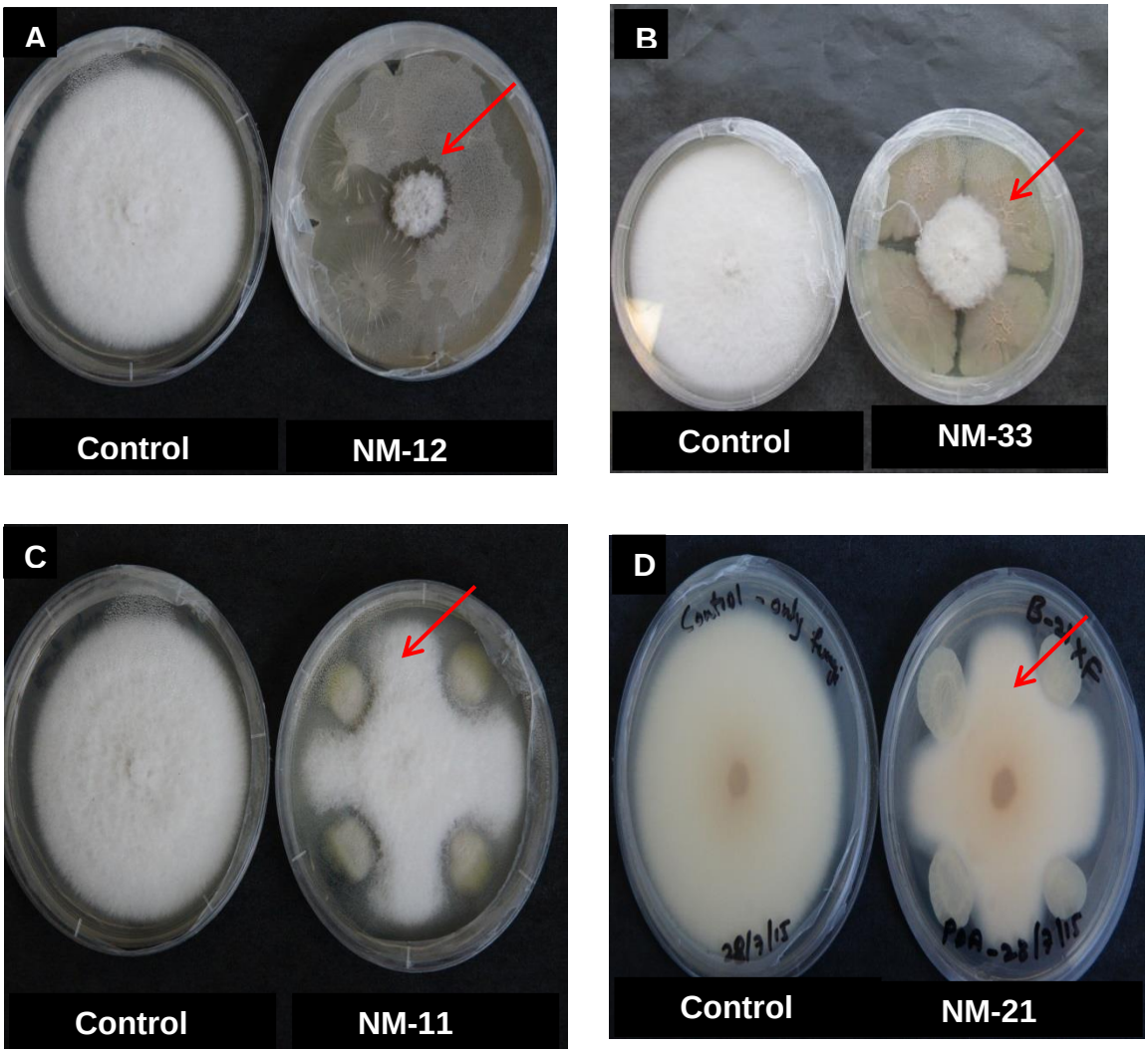
Figure 3.1: Rapid isolation of plant growth promoting bacteria plates (A) and B) Bacterial and fungal colonies. Red circle shows the small zones of clearance within the plate, C) Isolation plate without fungal spores, D) Established pure bacterial strains, E) Agar slants for culture maintenance)

The bacteria isolated from Australian agricultural soils were examined using a rapid isolation and screening approach. During the isolation, fungal spores were first plated on the agar followed by inoculation with the bacterial consortium from Australian soils. Strains which produced small zones of clearance (Figure 3.1 A, 3.1B) were picked and pure cultures were made by sub-streaking. Not many bacteria grew on soil extract agar during the isolation process but tryptone soy agar and nutrient agar both provided a vast diversity of bacteria. Maximum diversity of bacteria was observed on tryptone soy agar. Interestingly, all the effective bacterial isolates which produced zone of clearance were from Perry Bridge, Victoria, Australia. Based on the clear zones around the bacteria, single colonies were picked and subcultured on tryptone soy agar and nutrient agar to obtain pure cultures and selected for secondary screening.

3.3.2 Dual culture assay for identification of antagonistic bacteria

A dual culture assay was used to determine the capacity of the 32 bacterial isolates to inhibit the fungal pathogen following elimination of sibling strains (from the same species). The results of the dual culture assay showed that the bacteria varied in terms of their ability to supress radial growth of the fungus (*F. pseudograminearum*) as compared to the control. Four bacterial strains showed promising antifungal activity by inhibiting *Fusarium*. Two strains showed significant suppression (Figure 3.2A, 3.2B) whereas two other strains partially suppressed the fungus (Figure 3.2C, 3.2D).

Strain NM-12 demonstrated the best results in terms of reducing the radial growth of the fungus (1.6 cm) as compared to the control (8.4 cm). Strain NM-33 was the second most promising strain as it restricted the radial growth of the pathogen to 2.6 cm. Further elaborating on the results, it could be seen that NM-12 caused 80% inhibition whereas NM-33 caused 67% inhibition (Figure 3.3A, 3.3B). Only the aggressive strains (NM-12 and NM-33) were considered for further evaluation in this study.



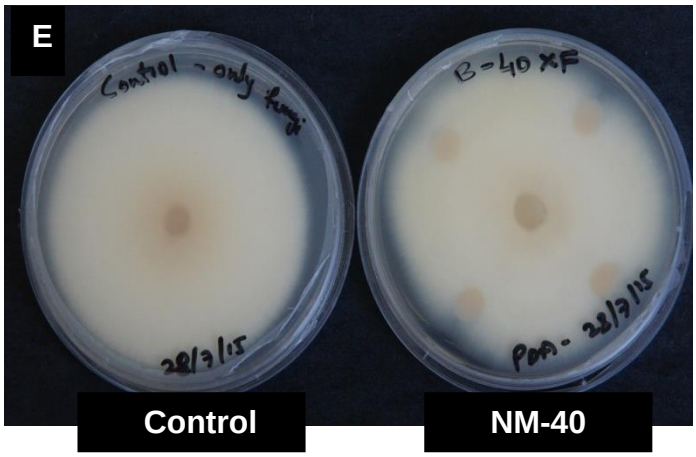


Figure 3.2: Dual culture assay of bacterial isolates and Fusarium pseudograminearum on potato dextrose agar plates

Control (fungus only) & NM-12 (arrow shows suppression of the fungus)

A. Control (fungus only) & NM-33 (arrow shows suppression of the fungus)

B. Control (fungus only) & NM-11(arrow shows partial suppression of the fungus)

C. Control (fungus only) & NM-21(arrow shows partial suppression of the fungus)

D. Control (fungus only) & NM-40 E) no activity against the fungus

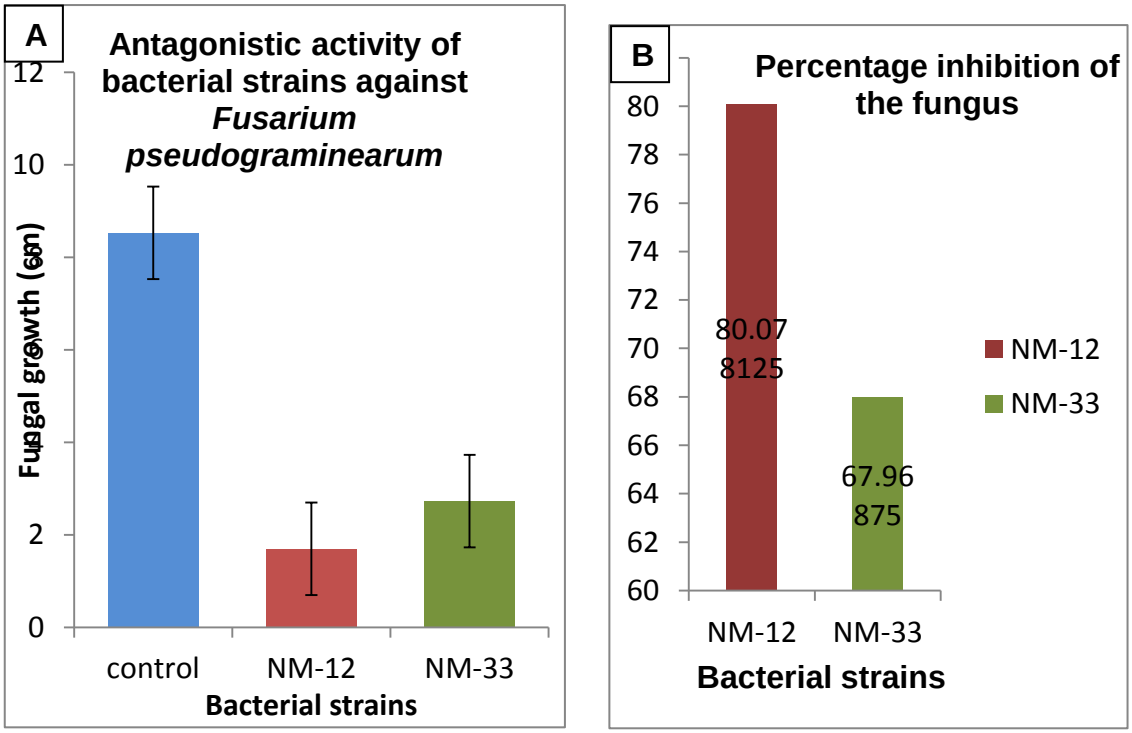
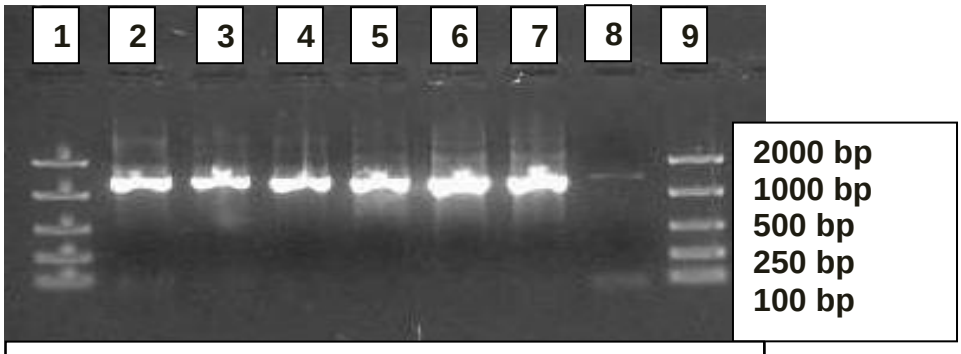


Figure 3.3: Suppression of fungal growth by bacterial isolates NM-12 and NM-33. (A. radial growth of fungus in presence and absence of the bacterial isolates, B. Percent inhibition of fungus after 7 days incubation at 28oC)

The variation in the ability of bacterial strains to inhibit the pathogen may be due to the strain type and the type of antifungal compounds that are released by the isolates in the culture media (AI-Azawi and Nawar 2010). For example, Grosu et al. (2015) found that a *Bacillus* spp. strain inhibited *F. graminearum* schwabe (which also causes crown rot) and *F. culmorum*. This strain was more potent compared to a *Bacillus amyloliquefaciens* strain. This was attributed to the ability of *Bacillus spp*. to produce glucanase, chitinase and ilutirin antifungal compounds. Therefore, the NM-12 and NM-33 isolates from this study were further characterised to determine if they had the ability to secrete antifungal compounds using a cell filtrate assay.

3.3.3 16S rRNA sequencing to identify the two antagonistic bacterial strains

The 16S rRNA gene was successfully amplified from the two bacterial strains, NM-12 and NM-33. As seen in Figure 3.4, the expected bands of 16S rRNA gene were obtained and sent over for sequencing.



Lanes 1 & 9: Ladder (Bioline EasyLadder I)

Lanes 2, 3, 4: NM-12

Lanes 5, 6 7: NM-13

Lane 8: Negative control

Figure 3.4: PCR gel image of 16s rRNA amplification

The 16S rRNA sequencing indicated that the bacterial isolates aggressive in suppression of *F. pseudograminearum* were most closely related to *Bacillus subtilis* and *Stenotrophomonas rhizophila.* The sequence analysis revealed that NM-12 strain was highly genetically similar (99%) with *Bacillus subtilis* and the NM-33 strain showed 97% similarity with *Stenotrophomonas rhizophila.* The phylogenetic tree is shown in Figure 3.5. These results show that strains NM-12 and NM-33 belong to the *Bacillaceae and Xanthomonadaceae* family that contain several N_2-fixing organisms (Reinhardt et al., 2008). It is reported that rhizosphere bacteria are largely represented by *Bacillus, Pseudomonas* and *Xanthomonas* strains in the rhizosphere of legume plants (Bent, 2006).

Table 3.7: 16S rDNA sequences (5'- 3') of bacteria isolated from Australian soils

STRAINS
>NM 12 **TGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGA** **TGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCG** **GCGCATTAGCTAGTTGGTGAGGTAACGGCTCCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGT** **GATCGGCCCMCACTGGGACTGAGACACGGCCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATYTTC** **CGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTC** **TGTTGTTAGGGAAGAACAAGTGCCGTTCGAATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCC** **ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGC** **GTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCAT** **TGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAG** **AGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC**

GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGG

GGGTTTCCGCCCCTTAGTGCTGCACTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACT

GAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAGCAACGCG

AAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAG

TGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAARTCCCSCAACGAGCGG

CAACCCTTGATCTTAKTTGCMAGCATTCMRTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG

GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACA

GAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAG

TCTGCAACTCGACTG

>NM-33

GACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGATAACGTAGGGAAACTTACGCTAATAC

CGCATACGACCTTCGGGTGAAAGCAGGGGACCTTCGGGCCTTGCGCGGATAGATGAGCCGATGTCGG

ATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCA

GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG

GGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTTTGTTG

GGAAAGAAAAGCAGTCGATTAATACTCGGTTGTTCTGACGGTACCCAAAGAATAAGCACCGGCTAACT

TCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTG

CGTAGGTGGTTGTTTAAGTCTGTTGTGAAAGCCCTGGGCTCAACCTGGGAATTGCAGTGGATACTGGG

CGACTAGAGTGTGGTAGAGGGTAGTGGAATTCCCGGTGTAGCAGTGAAATGCGTAGAGATCGGGAGG

AACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACACTGAGGCACGAAAGCGTGGGGAGCA

AACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGC

ACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAA

AGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCT

TACCTGGTCTTGACATGTCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAACACAGGT

GCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTG

TCCTTAGTTGCCAGCACGTAATGGTGGGGAACTCTAAGGGARACCGCCCGGTGACAAACCGGGAGGA

AAGGTGGGGGATGAACGTCCAAGTCCATCATGGGCCCTTTACGAACCAGGGGCTACACCACGTTACT

AACAATGGGTTAGGGACAGAGGGCTGCAAACCCGCGAGGGCAAGCCAATCCCAGAAACCCTATCTCA

GTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTC

Bacillus subtilis is a well-known plant-growth promoting bacteria and it has recently been reported that *Bacillus simplex* also exhibits antifungal activity when inoculated with *Rhizobium leguminosarum* during the growth of pea plants (Schwartz et al., 2013). In addition, *Stenotrophomonas* have an important role in the nature element cycling (Berg, 2009). A recent study has indicated that *Stenotrophomonas rhizophila* DSM14405 is of special agricultural interest in high saline conditions because it protects tomato, cotton and sweet pepper, probably by altering the fungal communities in the rhizosphere (Schmidt et al., 2012).

Previous studies also reported *Bacillus subtilis* as a biocontrol agent against *F. verticilliodies*, *F. graminearum* and *F. culmorum* (Cavaglieri et al., 2005; Grosu et al., 2015). Further, *Stenotrophomonas rhizophila* showed 90% *in vitro* antagonism against *F. oxysporum* (Adame-Garcia et al., 2015). Similarly, in this present study, *Bacillus subtilis* (NM-12) and *Stenotrophomonas rhizophila* (NM-33) showed significant antagonism when co-cultured with *F. pseudograminearum.* Thus, antagonistic bacteria such as those reported in this study might have the potential for reducing the incidence of FCR in crown rot infected regions of Australia. Further characterisation of these bacteria by sequencing would reveal if they are similar or different to the beneficial bacterial strains reported earlier.

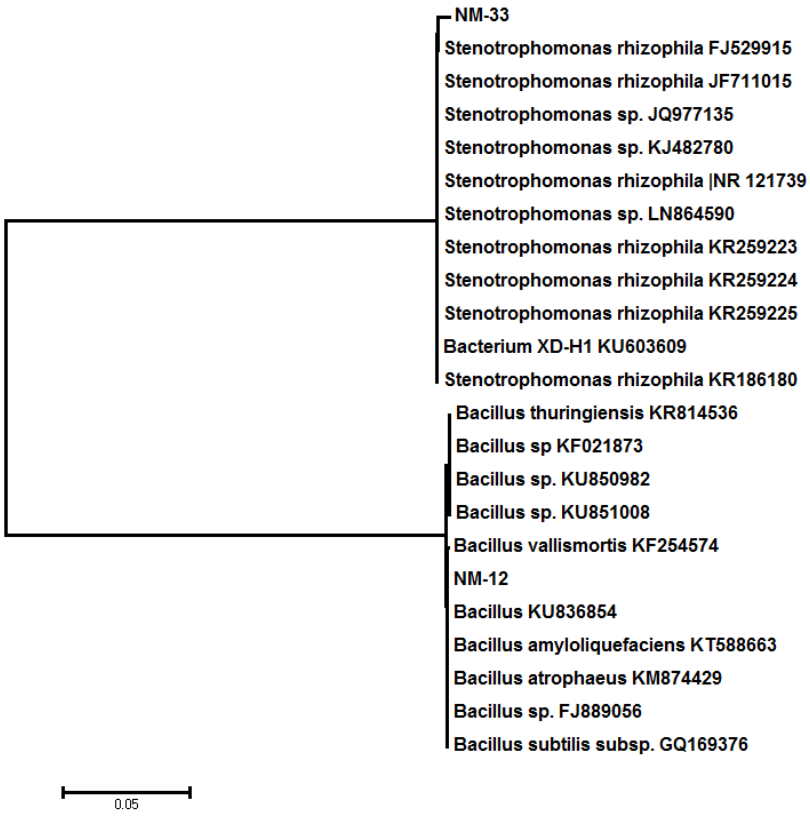


Figure 3.5: Phylogenetic tree obtained from Clustal W.

From the phylogenetic tree, it was inferred that the strains NM-12 and NM-33 shared similarities with *Bacillus and Stenotrophomonas* species

3.3.4 Culture filtrate assay on agar plates

A culture filtrate assay was used to determine if the antagonistic bacterial isolates were capable of secreting anti-fungal compounds. Bacterial strains that showed aggressive suppression of fungus during the dual culture assay were assessed further using cell culture filtrate assay.

The fungal mycelia in the control plate grew all over the plate (8.4 cm radially) during the 7 days of incubation, whereas the fungal growth in the culture filtrate amended agar was significantly suppressed. The culture filtrate of NM-12 only allowed the fungus to grow by 1.8 cm radially whereas the NM-33 filtrate checked

the fungal growth to 2.1 cm (Figure 3.6A, 3.6B). The bacterial strains supressed the growth of *F. pseudograminearum* during their stationary phase (third day of incubation).

The results from the assay show that the cell supernatant (culture filtrate) from the effective bacterial strains, NM-12 (*Bacillus spp*.) and NM-33 (*Stenotrophomonas rhizophila*), suppressed the fungal pathogen with 79% and 74% efficiency, respectively (Figure 3.7). The suppression is perhaps due to secondary metabolites present in the cell filtrate such as fungal cell wall degrading enzymes (Naglot et al, 2015), antibiotics and other antifungal compounds. While trying to explain the difference between inhibition percentages of both species, it can be said that the percentage depends on the type of species (Naglot et al, 2015).

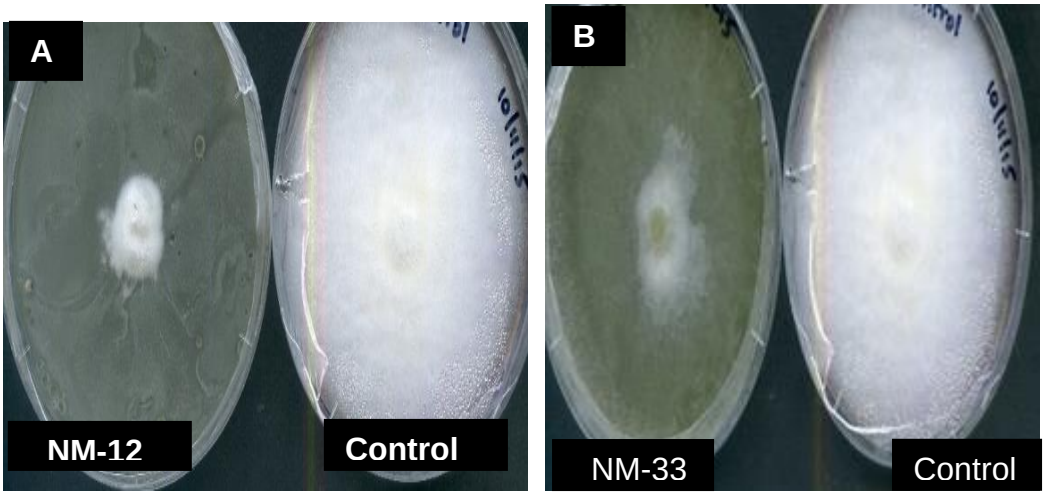


Figure 3.6: Effect of bacterial cell culture filtrates on the growth of Fusarium pseudograminearum in PDA (A. NM-12 cell filtrate with fungus & control (fungus only), B. NM-33 cell filtrate with fungus & control (fungus only)

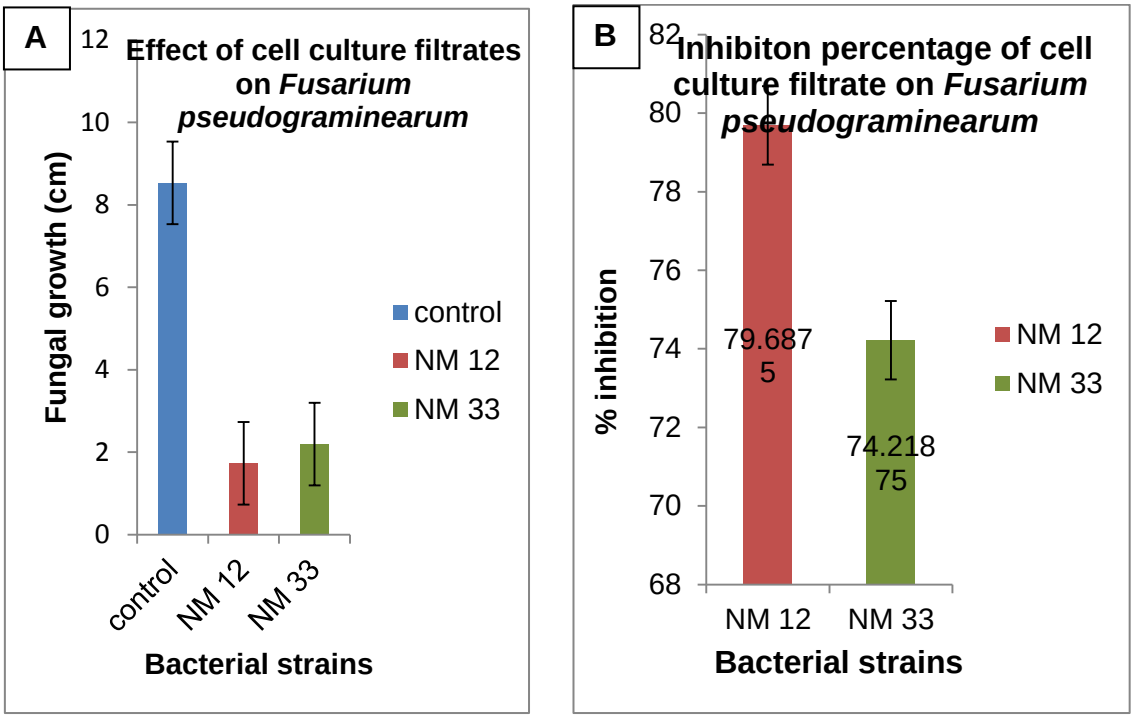


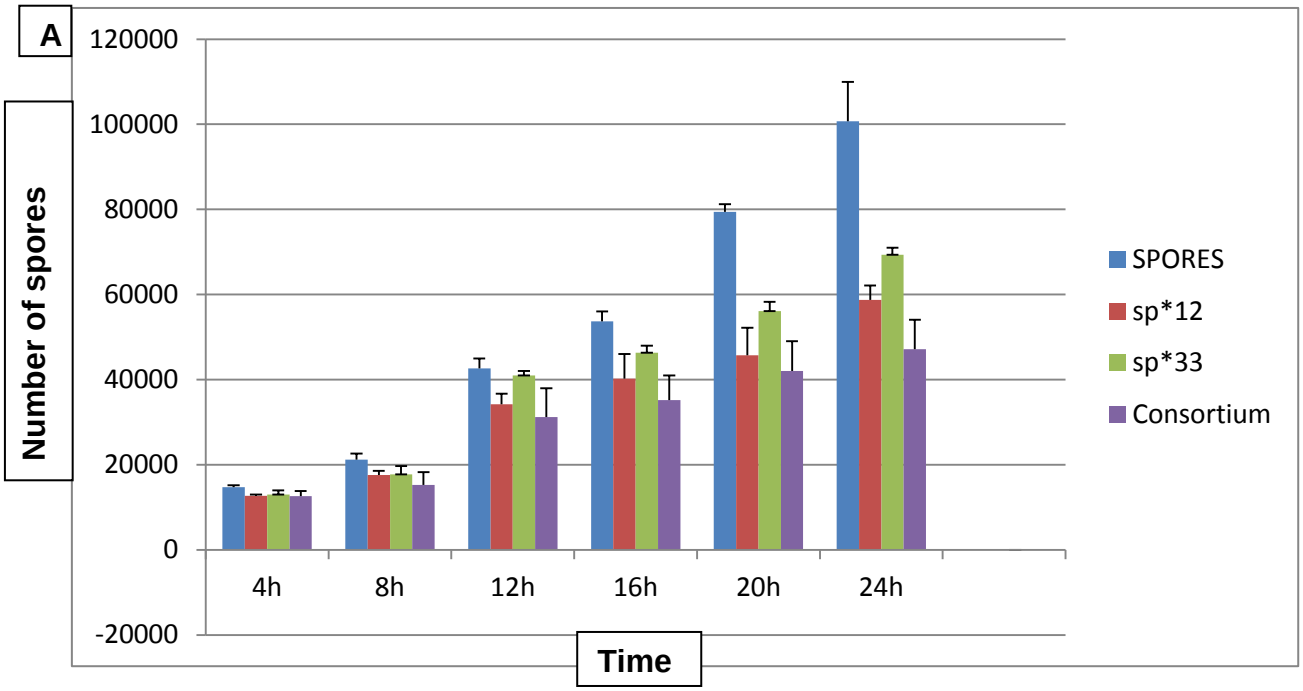
Figure 3.7: Influence of the culture filtrate from bacterial isolates NM-12 and NM-33 on fungal growth (A. Radial growth of fungus after seven days in presence and absence of cell filtrates, B. Percent inhibition of fungus after 7 days incubation at 28oC)

Naglot et al. (2015) found that a strain of *Trichoderma* species isolated from rhizosphere soils of tea gardens showed maximum antagonistic activity on *Pestalotia theae* with a 70% reduction in radial growth, obtained during the stationary phase of growth. The antagonistic activity was reportedly due to extracellular metabolites and enzymes such as chitinases and proteinase K. Similarly, antagonistic activity of *Bacillus* and *Stenotrophomonas* species against fungal phytopathogens has been reported by several other researchers (Indira 2011). *Bacillus* and *Stenotrophomonas* spp. in particular have been reported to exhibit substantial variability among strains in terms of antifungal abilities and host type (Chet 1989). Therefore, the strains reported in this study may potentially have better biocontrol activity against *F. pseudograminearum*.

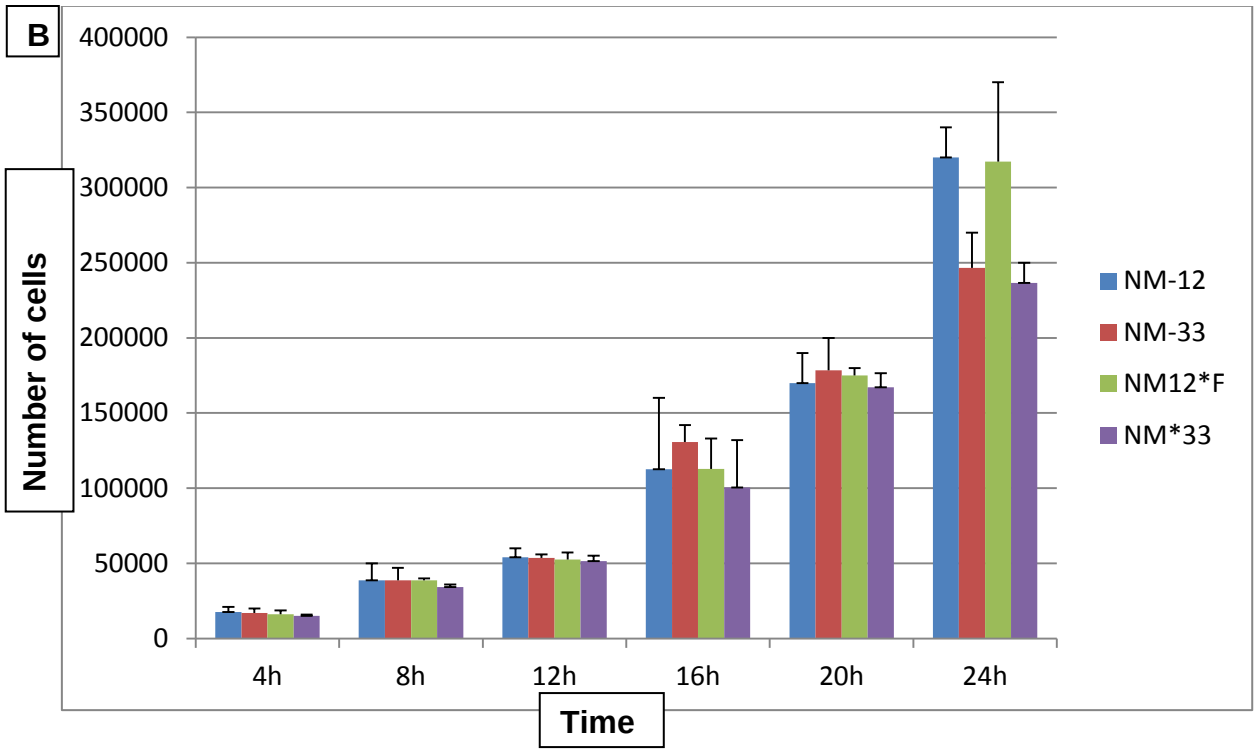
The two assays described above were performed to determine the biocontrol activity against the economically important fungal pathogen *F. pseudograminearum*. Among the antagonists, the strain designated as *Bacillus subtilis* (NM-12) showed higher inhibition efficiency in the culture filtrate inhibition assay, suggesting that the strain NM-12 (*Bacillus subtilis*) and NM-33 (*Stenotrophomonas rhizophila*) exhibit broad spectrum biocontrol activity which is quite substantial.

3.3.5 Antifungal activity assessed using by cell count in broth using a haemocytometer

Equal concentration (20 µL of 10^4 cfu/mL) of selected strains (NM-12, NM-33 and co-inoculation of both strains) and fungal spores were inoculated into the GY broth. Cultures were incubated on a shaker at 28 ^oC for 24 h to attain maximum sporulation of fungus and bacterial cell division. The bacterial cell count and fungal spore count were monitored every 4 hours and compared with the control (only fungus or bacteria) as a reference. As seen in Figure 3.9A, a combination of both strains (NM-12 and NM-33) resulted in the highest reduction in the fungal spore count (down to 4.71 x 10^4 spores/mL) as compared to control (10.73 x 10^5 spores/mL). In comparison, strains NM-12 and NM-33 reduced the spore count of the pathogen to 5.87 x 10^4 and 6.93 x 10^4 spores/mL, respectively. Interestingly, when comparing the growth of bacterial strains with and without fungus, there was no significant difference at all time points (Figure 3.8B). This suggests that the fungus was not capable of affecting bacterial growth in the liquid media.



Fungal spore count



Bacterial cell count

Figure 3.8: The antifungal activity of the two bacterial isolates NM-12 and NM-33 and a mixture of both strains during co-culture in liquid medium for 24 h. fungal spores (A) and bacterial cells (B) were enumerated as number per mL.

The development of the dual culture broth assay to determine the inhibition of fungal growth represents an approach developed specifically for this project. To the best of my knowledge this study is the first to attempt to understand the inhibition effect of bacteria on fungal growth in liquid culture by inoculating equal amounts of bacterial and fungal cells.

3.3.6 Antagonistic activity by fungal biomass dry weight

The growth of fungus (*F. pseudograminearum*) in the presence of the antagonistic bacterial isolates and their consortium was further studied on GY media. The growth of mycelia was observed on the third day. Mycelial biomass was obtained by filtering pre-weighted filter paper (Whatman no.1) and drying in hot oven at 90^{o}C until a constant weight was achieved. The variation in the weight compared to the control suggested reduced biomass and growth of the fungus (Senthamizhlselvan et al., 2010). The experiment was performed in triplicate. Overall, analysis of the mean mycelial weight confirmed inhibition of *F. pseudograminearum* in the presence of bacterial isolates in GY media. Once again, the consortium consisting of both strains (NM-12 and NM-33) showed the highest activity in terms of reducing the fungal biomass (0.034 g/ML) as compared to the control (0.099 g/mL) (Figure 3.9). Comparatively, in the presence of individual strains NM-12 and NM 33, the fungal dry weights were 0.049 g/mL and 0.054 g/mL, respectively. This again suggests that both the antagonistic strains probably use somewhat different mechanisms for fungal suppression.

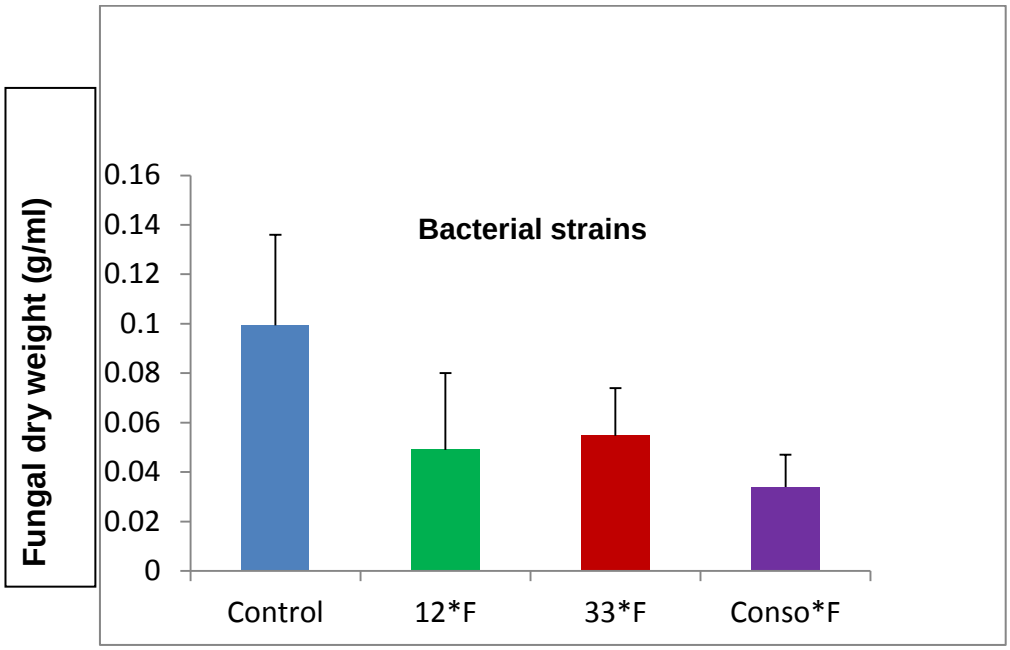


Figure 3.9: Determination of the antifungal activity of the two bacterial isolates, NM-12 and NM-33, and a mixture of both strains by co-culture with fungus in liquid medium for 24 h. fungal growth is expressed as g/ml dry weight.

3.4 CONCLUSIONS

Two bacterial strains that were able to inhibit the *in vitro* growth of *F. pseudograminearum* were identified in this study. These were identified as *Bacillus subtilis* (NM-12) and *Stenotrophomonas rhizophila* (NM-33) using 16S rRNA sequencing. NM-12 caused greater inhibition and supressed fungal growth in solid media as well as liquid broth. Interestingly, the consortium of the two strains together caused more inhibition than individual strains even when inoculated at the same concentration. This suggested somewhat different mechanisms used by these strains for fungal suppression. These mechanisms (production of antibiotics, chitinase, siderophore, hydrogen cyanide, etc.) are characterised in the next chapter.

4 CHAPTER 4: CHARACTERIZATION OF PLANT GROWTH PROMOTING BACTERIA IN TERMS OF SECONDARY METABOLITE PRODUCTION

4.1 INTRODUCTION

Plant growth promoting bacteria (PGPB) have been and remain the most successful source of microorganisms for various bioactive metabolites, including those involved in the biocontrol of pests and diseases. The capacity of these bacteria to produce extracellular enzymes has long been an area of research interest, but there has been a recent spike in interest in this research due to the search for environmentally friendly biocontrol agents (Gurung et al, 2013). Besides their ability to decompose organic matter, PGPB also promote plant growth by suppressing plant pathogens (Hoster et al. 2005; Thirup et al. 2001; Nassar et al. 2003). This is achieved by producing various primary and secondary metabolites including extracellular enzymes that have antimicrobial activity, siderophores and antibiotics. In addition they also produce substances that promote plant growth and solubilise phosphates, and compete with plant pathogens for substrates and nutrients (Cattelan & Hartel, 2000). The most studied PGPB, which are extensively promoted as biological control agents belong to five genera: *Bacillus, Streptomyces, Pseudomonas, Agrobacteria* and *Burkholderia* (Manero et al., 2003). Among all, *Bacillus* sp. are considered as the most potent due to their abundance in the rhizosphere, their spore-forming capacity and their ready adaptation to commercial formulations and field application (Ryu et al. 2004; Gajbhiye et al. 2010). One competent species, *Bacillus subtilis* has been reported to protect plants from pathogens thereby resulting in healthy growth and improved

yield. Another strain, *B. mucilaginous,* has been shown to solubilise potassium (Han et al. 2006).

Several species of the *Streptomycetaceae* family have been extensively studied in terms of their ability to produce secondary metabolites (antibiotics and extracellular enzymes). Interestingly, a recent study has established a relation that exists between epibiotic predation and antibiotic production in the genus *Streptomyces* (Kumbhar et al. 2014). Moreover over a thousand secondary metabolites have been identified to be produced by actinobacteria during the years 1988-1992. Importantly, almost all of these originated from soil isolates.

The interaction between plants and PGPB occur through either endophytic or symbiotic associations depending on their degree of proximity to the roots and surrounding soil (Souza et al. 2015). However, the real triggers that activate the biosynthesis of metabolites in PGPB are as diverse as their products. They vary from environmental cues (carbon, nitrogen, pH, etc.) to the presence of other microbes living in the same habitat (Brakhage, 2013). Several *in vitro* mixed cultivations have been shown to produce enhanced levels of secondary metabolites; thereby supporting the notion that co-cultivation of two or more selected bacterial strains on solid or liquid medium can enhance the synthesis of known metabolites (Tarkka et al. 2009; Marmann et al. 2014; Bertrand et al. 2014; Schroeckh et al., 2014). In addition, such cultivation allows rapid production of a desirable metabolite.

Even though the cultivation of pure or single strains is considered as the standard method for metabolite biosynthesis in modern natural product chemistry, the

growth conditions that are required by the single-strain cultures are extremely different as compared to the natural environment where a number of microbes interact with each other (Onaka et al., 2011). Moreover, pure strain cultivation may lead to a reduction in the number of metabolites produced and therefore may limit their observed commercial potential. Hence, new fermentation practices have been identified and developed that can help in overcoming the identified limitation, among which the method that is commonly used is the one that consists of co-cultivation of two or more microbes together (Zuck et al., 2011). Activation of complex regulation mechanisms, such as the antagonistic, competitive, facilitative and neutral outcomes are also said to get activated because of interactions that take place among microbes (Estrada et al., 2011).

A key reason that has been identified from studies is that production of bioactive secondary metabolites can be caused due to the microbial interactions (Bertrand et al, 2013a). Hence, harvesting of new molecules can be accomplished via the co-culturing of different microbial strains. As an example, researchers tried to isolate a new antibiotic, pestalone, from the mixed fermentation co-culture of a marine-derived Gram-negative bacterium of the genus *Thalassopia* sp. (CNJ-328) and the marine fungus *Pestalotia*; however the antibiotic was not found to be present when the culturation of the two strains was done separately. Pestalone displayed a strong antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (Cueto et al., 2001).

From all the *in vitro* studies conducted on PGPB to target the specific agents for pathogen control and plant growth, the production of hydrolytic enzymes, antagonistic activity against pathogens, the production of siderophores, the capacity to colonize the root system, and the production of plant growth regulating compounds all appear of vital importance (Cattelan, 1999). Furthermore, abiotic factors such as soil salinity and pH interfere with the competitive ability of these potential biological agents (Sousa et al. 2008). Hence, resistance towards high salinity or low pH becomes essential criteria for the selection of PGPB (Drozdowicz, 1987). In this chapter, the production of four different primary and secondary metabolites, indole acetic acid (IAA), siderophores, β-glucanase, and volatile components by the putative PGPB isolates, NM 12 and NM 33 were studied. Further, their production under saline conditions was evaluated to determine their ability to suppress pathogens and promote plant growth in saline soils. These two strains were selected on the basis of their demonstrated potential for plant growth promoting traits (Chapter 3).

The Objectives of these experiments were:

- To evaluate is strains NM-12 and NM-33 are capable of producing IAA, siderophore, and β-glucanase under normal condition and 4%, 6%, 8% and 10% salt concentrations.
- To test the effect of volatile components of NM-12 and NM-33 on the growth of *Fusarium pseudograminearum*
- To identify if the bacterial isolates have HCN and ACC deaminase genes.

4.2 MATERIALS AND METHODS

Screening of isolates for plant growth promoting properties and pathogen control

The experiments described in this chapter include evaluation of selected strains, NM-12 and NM-33 in terms of secondary metabolite production which encompasses IAA, siderophore, and β-glucanase. Subsequently, volatile compounds produced by these strains were evaluated for their ability to suppress the fungal pathogen, *Fusarium pseudograminearum*. The strains were sub-cultured from glycerol stocks onto agar slants and streaked on nutrient agar plates.

4.2.1 Indole acetic acid (IAA) assay

Principle: Hydrogen peroxide induces the oxidation reaction in IAA and produces a pink colour. It is specific for indoles. The Fe (III) present in Salkowsky's reagent chelates the two nitrogen atoms from the resulting condensation compound to give a variety of hues of colours based on the substituents of the indoles.

Procedure: The IAA production assay was performed according to the standardized protocol described by Gopalakrishnan et al. (2011). Starch-casein broth (SCB; Table 4.1) was amended with tryptophan and different NaCl concentrations of 4%, 6%, 8% and 10%. The significance of using tryptophan with SCB is that tryptophan acts as a precursor for IAA synthesis.

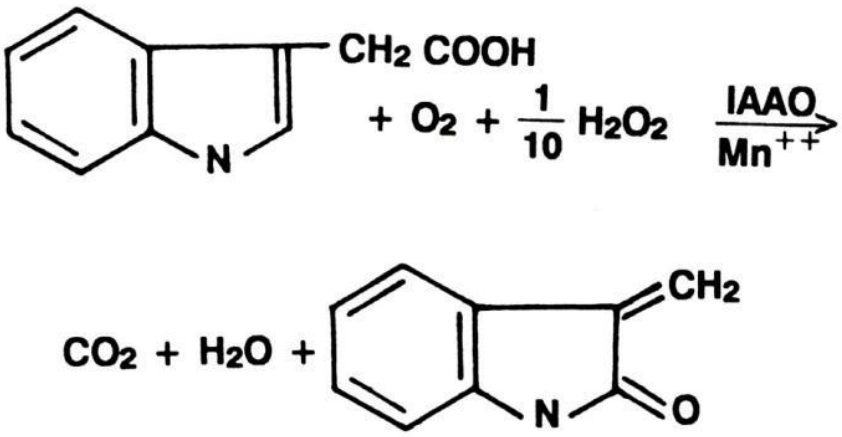


Figure 4.1: Oxidation of IAA

Item	Amount
CaCO3	0.02 g
FeSO4 .7H2O	0.01 g
KNO3	2.0 g
K2HPO4	2.0 g
MgSO4.7H2O	0.05 g
NaCl	2.0 g
Starch	1%
L-Tryptophan	1.0 g
Casein	0.3 g
Deionized water	1000 mL
pH	7.0

Table 4.1: Composition of Starch-casein broth (SCB)

Freshly grown strains of NM-12 and NM-33 were inoculated in 100 mL of SCB and incubated on a shaker for 5 days at 150 rpm. The cultures were subsequently centrifuged at 10,000 rpm for 12 min. An aliquot (2 mL) of Salkowsky's reagent was added to 1 mL of culture supernatant and incubated in the dark for 30 min. Salkowsky's reagent was prepared by dissolving 0.81 g of $FeCl_3$ in 10 mL of distilled water. A 1 mL aliquot of this solution was added to 500 mL of 35% $HClO_4$ to produce the final Salkowsky's reagent. The development of a pink colour indicated the presence of IAA. The amount of IAA produced by the cultures was estimated by measuring absorbance at 530 nm. Standards were prepared according to the Table 4.2.

Table 4.2: Preparation of standards for IAA estimation

IAA Conc (mg/mL)	**Working Stock (mL)**	**Distilled Water (mL)**	**Salkowsky's Reagent (mL)**	**Total Volume (mL)**
0	0	2	6	8
0.1	0.4	1.6	6	8
0.4	0.8	1.2	6	8
0.6	1.2	0.8	6	8

0.8	1.6	0.4	6	8
1	2	0	6	8

4.2.2 Siderophore assay

Principle: A siderophore is a strong iron chelator that separates the Fe (III) ion from its natural complex forming a siderophore-Fe complex. The principle can be explained by the following chemical equation.

Fe Dye (CAS-Chrome Azurole Sulphonate) + Siderophore → Fe-Siderophore + Dye

When the iron-siderophore complex is formed, the free dye released from this reaction causes a colour change from blue to orange.

Procedure: The procedure to evaluate the production of siderophores was as described by Macagnan et al. (2008). Initially, all glassware was rinsed with concentrated HCl to ensure that no traces of metal ions were present as the ions may interfere with the final reaction. King's B broth was prepared under normal and saline conditions with graded NaCl concentrations of 4%, 6%, 8%, and 10% (Table 4.3). The significance of using King's B is that it is an iron-free media and siderophores are produced only in iron starvation conditions.

Table 4.3: Composition of King's B broth

Item	Amount
Peptone	: 5.0 g
K_2HPO_4	: 1.2 g
$MgSO_4.7H_2O$	: 1.5 g
Glycerol	: 2 mL
Deionized water	: 1000 mL

The two strains, NM-12 and NM-33, were inoculated into King's B media and incubated at room temperature on a rotary shaker for 4 days at 150 rpm. The 4-day old cultures were centrifuged at 5,000 rpm for 12 min and the supernatants were collected. To 1 mL of the supernatant, an equal amount of CAS solution was added and incubated in the dark for 30 min.

CAS solution was prepared according to Schwynn and Neilands (1987).

- 10 mM HDTMA solution was prepared by dissolving 0.364 g of HDTMA in 100 mL distilled water.
- $FeCl_3$ solution was prepared by adding 16.2 mg of $FeCl_3.6H_2O$ in 3.6 mL of concentrated HCl and made up to 100 mL.
- CAS reagent was prepared by dissolving 0.12 g of CAS (chrome azurol S) in 100 mL distilled water.
- Pipes buffer was prepared by dissolving 4.307 g of anhydrous piperazine in how many mL distilled water. Then, 6.25 mL of 12 M HCl was

carefully added and the pH was adjusted to 5.6 by adding 100% NaOH (w/v) drop wise.

The final CAS solution was prepared by mixing 6 mL of HDTMA solution, 1.5 mL of $FeCl_3$ solution, 7.5 mL of CAS reagent and Pipes buffer under constant stirring conditions. The final volume was made up to 100 mL in a standard flask with distilled water. The final CAS solution appeared blue and was stored in a brown bottle to avoid light exposure.

The presence of siderophores is indicated by a colour change from blue to orange. The amount of siderophores produced was calculated by measuring the absorbance values at 630 nm. The percentage of siderophore units was calculated using the formula:

% Siderophore units = A_S-A_r/A_r×100

Where,

Ar = Reference OD value (uninoculated media + CAS solution)

As = Sample value (culture supernatant + CAS solution)

4.2.3 β-glucanase assay

Principle: β-glucanase cleaves laminarin, a glucan polymer releasing glucose molecules. One unit of β-1, 3 glucanase activity is defined as the amount of enzyme required to liberate 1 µmol of glucose per hour under defined conditions (Singh et al, 1998). By estimating the amount of glucose released, β-glucanase activity can be determined.

Procedure: The protocol for the β-glucanase assay was standardized by Singh et al. (1999). The production of β-glucanase enzyme was tested by inoculating NM-12 and NM-33 strains individually into tryptone soy broth (TSB; Table 4.4) amended with NaCl concentrations of 4%, 6%, 8% and 10% together with 1% colloidal chitin. The significance of using chitin with TSB media is that it is a glucose-free medium in which chitin is the sole source of carbon. The presence of glucose in the media interferes with the evaluation of the β-glucanase activity.

Table 4.4: Composition of Tryptone soy agar

	Item	Amount
	Tryptone	17.0 g
	Soybean meal	3.0 g
	NaCl	5.0 g
	K_2HPO_4	2.5 g
	pH	7.3±0.2

Cultures were kept on a rotary shaker for 4 days at 180 rpm. Four-day old cultures from each flask were centrifuged at 10,000 rpm for 12 min and the supernatants were collected. To 1 mL of the supernatant, 0.1 mL of laminarin (2% wt/vol) was added and then incubated at 40°C for 1 h. During this incubation period, β-glucanase cleaves the substrate laminarin producing glucose molecules. The reaction was stopped by adding 3 mL of dinitrosalicylic acid (DNS) reagent and incubating tubes in a boiling water bath for 10 min. The colour transition from yellow to red indicated the presence of glucose, demonstrating the

activity of β-glucanase. The concentration of glucose produced by the cultures was estimated by measuring the absorbance at 530 nm and determining the concentration through a calibration curve (Table 4.5).

Table 4.5: Preparation of glucose standards

Conc of glucose (mg/mL)	Working stock (mL)	Na-acetate buffer (mL)	DNS reagent (mL)	Total volume (mL)
0	0	2	6	8
0.2	0.4	1.6	6	8
0.4	0.8	1.2	6	8
0.6	1.2	0.8	6	8
0.8	1.6	0.4	6	8
1	2	0	6	8

4.2.4 Volatile components assay

The volatile components assay was performed according to the method described by Nakarin et al. (2012). Both strains NM12 and NM-33, were streaked on Bennet agar (Table 4.6) plates. The cultures were incubated at 30℃ for 7 days.

Table 4.6: Composition of Bennet agar

Item	Amount
Beef extract	1.0 g

Glucose	10.0 g
Casein enzyme Hydrolysate	2.0 g
Yeast extract	1.0 g
Agar	15.0 g
Deionized water	1000 mL
pH	7.3

A 5 mm disc of actively growing *F. pseudograminearum* was placed on potato dextrose agar (PDA; Table 4.7) plates. The lids of 7 day old NM-12 and NM-33 culture plates were removed and inverted on freshly placed fungal disc plates. The plates were double taped together with paraffin (Figure 4.1). Fungal plates not exposed to the volatiles of these strains were considered as controls. The diameter of the fungal zones was measured at 24 h intervals and the percentage of inhibition was calculated.

Table 4.7: Composition of Potato dextrose Agar

Item		**Amount**
Potato dextrose broth	:	24 g
Agar	:	2 %
Deionised water	:	1000 ml

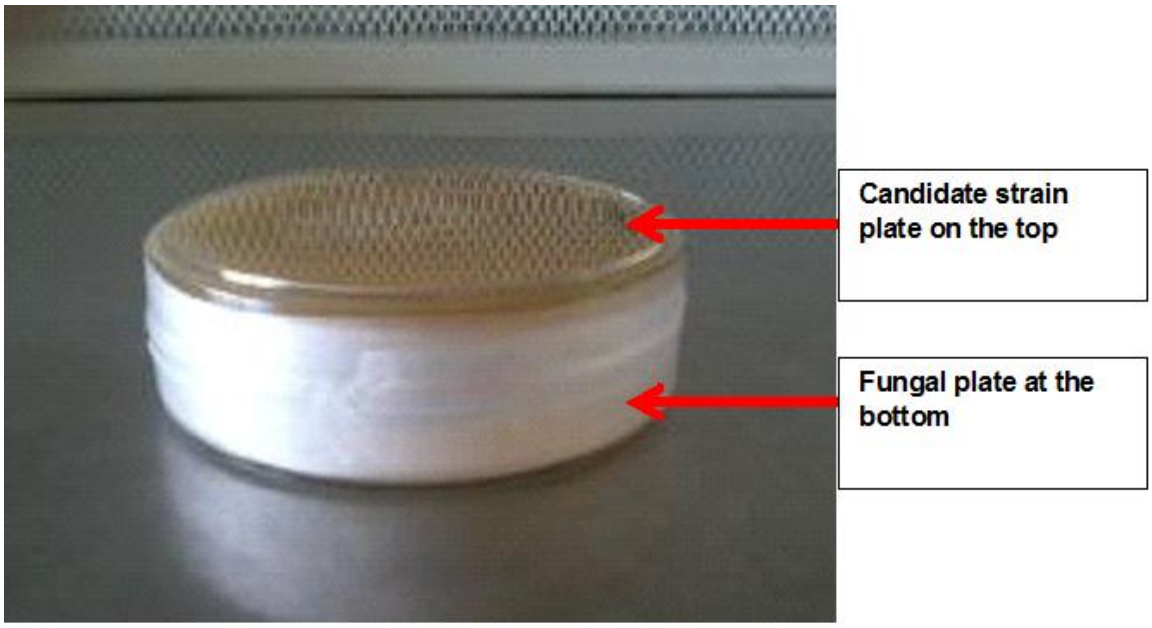


Figure 4.2: Candidate strain plate inverted on the fungal plate and double taped with paraffin

4.2.5 Identification of ACC deaminase and HCN producing genes

To assess the presence of ACC deaminase and HCN producing genes within the genome of the most promising isolate, NM-12, PCR reactions were performed to amplify the ACC deaminase and HCN producing genes.

Primer name	Target	Sequence (5' to 3')
F	ACC	ACCTGTTTGGAGATGGCGAG
R	ACC	AAGGAAGTTGCCCGTGTTCT
F	HCN	GAAACAGATTGCGGCGGATG
R	HCN	ATATACTGATCGCTCGGCGG

The 25 µL PCR mixture contained 2.5 µL of KOD hot start DNA Polymerase, 10 µL PCR buffer (20 mM Tris-HCl pH 7.5, 7.5 mM DTT, 50 µg/mL BSA), 2.5 µL of 2 mM dNTPs, 2 µL of 20 pmol each primer and approximately 100 ng template DNA. All reactions were performed in a MJ Instruments PTC-100 thermo cycler (Waltham, MA) with the following program: 1 minute and 30 seconds initial denaturation at 94°C, 35 cycles of 1 minute denaturation at 92°C, 50 seconds primer annealing at 58°C, 1 minute of elongation at 72°C. A final elongation step of 5 minutes at 72°C was included.

4.3 RESULTS AND DISCUSSION

In the present study, the production of primary metabolites namely, IAA, siderophores and β-glucanase from strains the NM-12 (*Bacillus subtilis*) and NM-33 (*Stenotrophomonas rhizophila*) under normal and stressed saline conditions was investigated. The effect of volatile compounds from these strains on the fungal growth inhibition of *Fusarium pseudograminearum* was also studied to confirm the pathogen control activity.

β-glucanases have been considered important in the biological control of soil borne pathogens because of their ability to degrade fungal cell walls, of which β-glucan is an important component. It was earlier reported that *Bacillus subtilis* produced extracellular hydrolytic enzymes such as β-1, 3 glucanase (Prapagdee et al., 2008). In the present study, the production of β-glucanase was observed in normal and saline conditions also.

4.3.1 IAA production

By producing plant hormones, the PGPB stimulate plant growth by inducing the production of plant metabolites that are beneficial to their growth (Gopalakrishnan et al, 2011). Tryptophan is the main precursor for IAA biosynthesis in bacteria (Patten and Glick, 1996). A *Streptomyces* isolate, *Streptomyces ablidoflavus* was found to produce IAA in culture medium supplemented with L-tryptophan (Narayana et al, 2009). Tryptophan induces IAA production by the two strains, NM-12 and NM-33. The maximum production of IAA was seen by NM-12 strain under normal conditions. This is indicated by the appearance of a pink colour (Fig. 4.2). No IAA production was observed in strain NM-33 (Fig. 4.2). Isolate NM-12 produced 285.5 µg/mL IAA in tryptophan-supplemented media under normal conditions. However, IAA production in this strain decreased sharply with increasing NaCl concentrations irrespective of the presence of tryptophan (Figure 4.3). In 4% NaCl-supplemented media, IAA production by strain NM-12 decreased to 41.5 µg/mL which dropped further to 14.5 µg/mL in 6% NaCl-supplemented media. In comparison, strain NM-33 did not show any significant IAA activity.

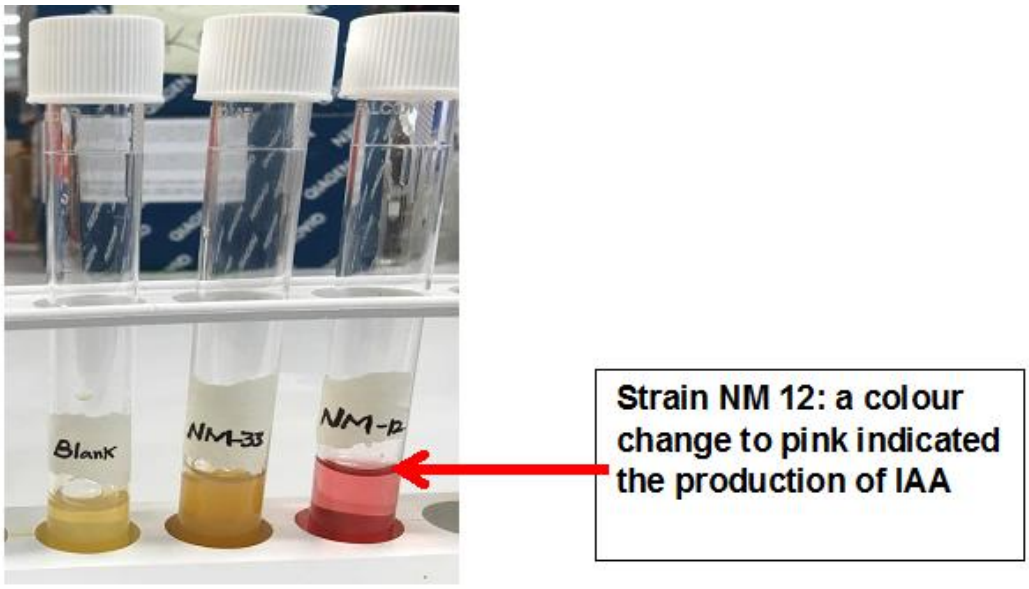


Figure 4.3: IAA production by NM-12 and NM-33 isolates under normal growth conditions.

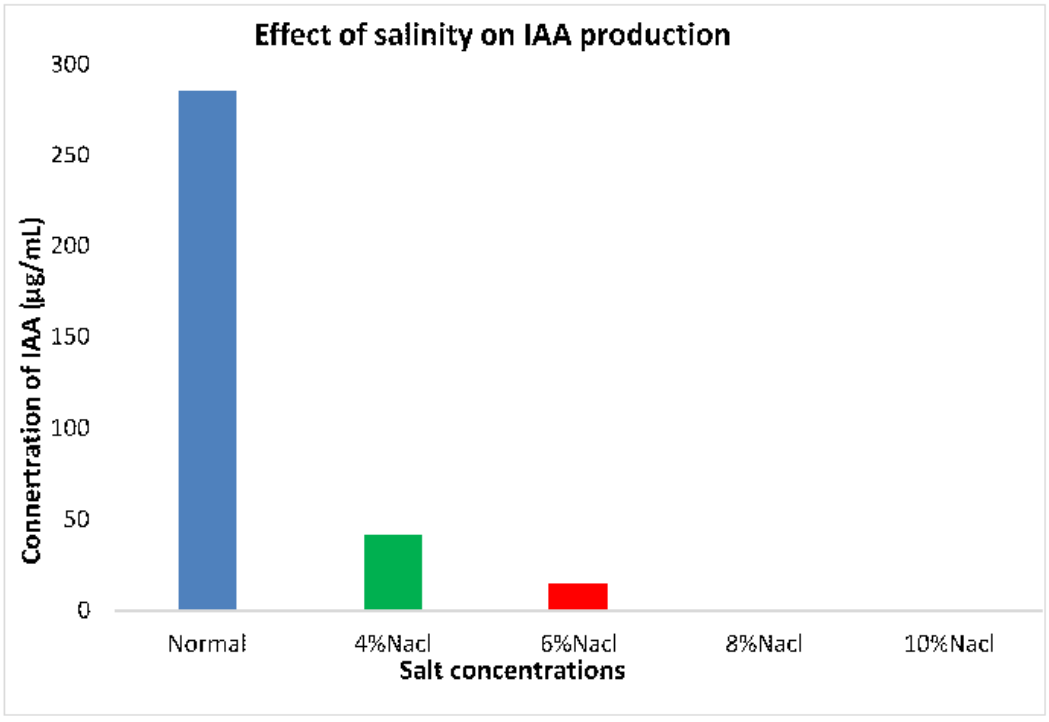


Figure 4.4: Influence of salt on IAA production (µg/mL) by strain NM-12

Table 4.8: Table 4.8 Absorbance values of cultures producing IAA at 530nm

Estimation of IAA:					
Isolate	**Normal salinity**	**4%**	**6%**	**8%**	**10%**
NM-12	1.412	0.190	0.074	-	-
NM-33	-	-	-	-	-

To estimate the amount of IAA produced by the two strains, the above OD values were plotted on the standard graph.

Table 4.9: Table 4.9 Standard OD values

Concentration (µg/mL)	**O.D. at 530nm**
10	0.027
20	0.077
30	0.134
40	0.19
50	0.244

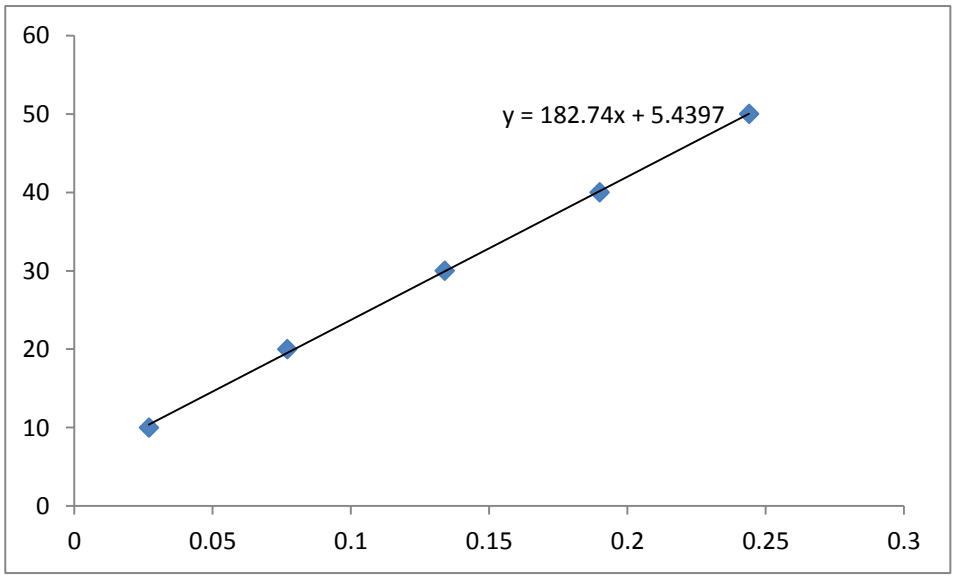


Figure 4.5: The calibrated graph according to the standard OD values:

4.3.2 Siderophore production

Siderophores are the low-molecular-weight molecules, which are usually secreted by the organisms that are present in iron-limiting conditions (Sadeghi et al., 2012). They complex environmental iron thereby depriving any plant pathogen of this essential mineral (Kloepper et al., 1980). According to Neilands (1995), siderophores are produced only under conditions of low iron availability. Nevertheless, in the current experiment, siderophores were produced under normal as well as saline conditions. Surprisingly, the production of siderophores increased with increasing salt concentrations, up to 6% NaCl concentration and thereafter declined gradually. The highest amount of siderophore production was observed in NM-12 and NM-33 at 6% salinity level (Fig 4.4). At 6% salinity, the concentration of siderophores (69.5 units) produced by NM-12 strain was almost twice the amount produced in non-saline conditions. Comparatively, strain NM-33 only increased siderophore production by half at 6% salt concentration. This demonstrated that NM12 has superior siderophore producing capacity in both saline and non-saline conditions compared to NM-33.

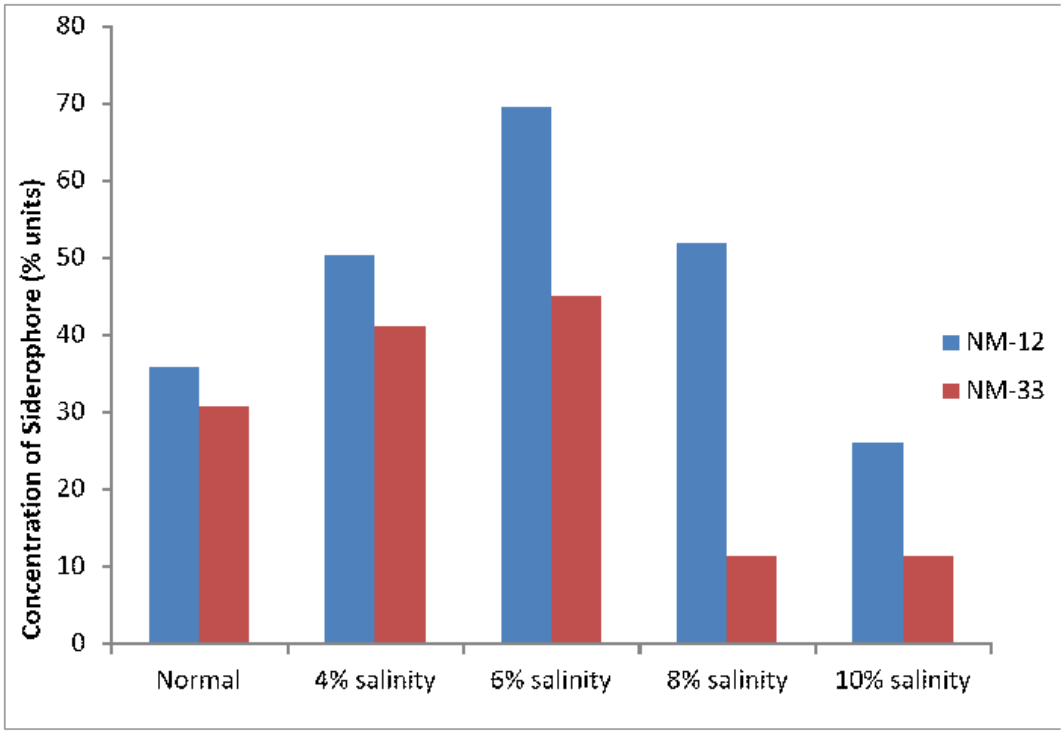


Figure 4.6: Siderophore production (%) by selected isolates, NM-12 and NM-13 under normal and stressed (saline) conditions

Previous studies have highlighted the importance of the genus *Stenotrophomonas* in siderophore synthesis. A number of studies showed that *Stenotrophomonas maltophilia* can synthesize different types of siderophores as assessed using the CAS assay. The production of ornibactin-type siderophore by *S. maltophilia* was reported by Chhibber et al. (2008). These strains have a close association with plants, promoting their growth and health (Ryan et al., 2009). The regulation of genes involved in plant growth promoting activities by *Stenotrophomonas rhizophila* DSM14405 have also been observed (Alavi et al., 2013). Similarly, different strains of *Bacillus* sp. have been reported for their capacity to produce siderophores (Gardner et al., 2004; Wilson et al., 2010).

4.3.3 β-glucanase production

β- Glucanases have been considered as an important biological control agent for soil-borne pathogens primarily because they are able to result into degradation of the fungal cell walls. It was earlier reported that *Bacillus subtilis* produced the extracellular hydrolytic enzymes

β-1 and 3 glucanases (Prapagdee et al., 2008). In the present study, isolates NM-12 and NM-33 were tested for the production of β-glucanase under normal and saline conditions (Fig. 4.5). Strain NM-33 showed greater β-glucanase activity in both normal and saline conditions. The strain NM-12 exhibited almost similar activity under normal and saline conditions. The production of β- glucanase from the NM-12 isolate was recorded as 252.3 units of activity under control conditions and 10% salinity but it showed maximum activity at 4% salinity (302.7 units) (Fig. 4.6). In comparison, strain NM-33 showed maximum enzyme activity (454.15 units) at normal condition and 353.23 units at 10% salt concentrations. Interestingly, the ability of both these strains to produce β- glucanases was similar at 4, 6 and 8% salt concentrations.

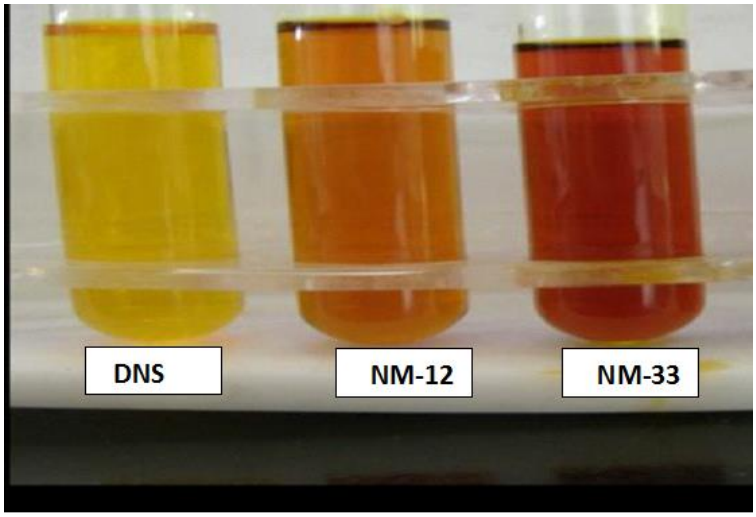


Figure 4.10: Glucose production under normal and saline conditions as seen by the development of red colour in NM-12 AND NM-33 tubes compared to the control (DNS)

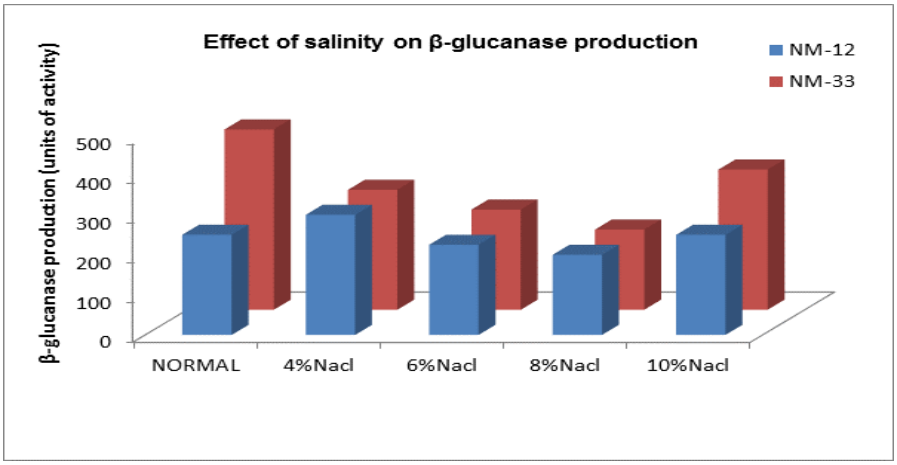


Figure 4.11: A comparison of β- glucanase production (units) by the two bacterial isolates, NM-12 (blue) and NM-33 (red) under normal and saline conditions

Table 4.10: OD values of the two strains at 530nm

Isolate	Normal salinity	4%	6%	8%	10%
NM-12	0.211	0.225	0.178	0.165	0.193
NM-33	0.378	0.236	0.198	0.185	0.248

The above OD values were plotted on the standard graph to estimate the amount of glucose.

Table 4.11: Standard OD values

Concentration	O.D at 530nm
0.2	0.551
0.4	1.635
0.6	2.358
0.8	3.612

1.0	3.913

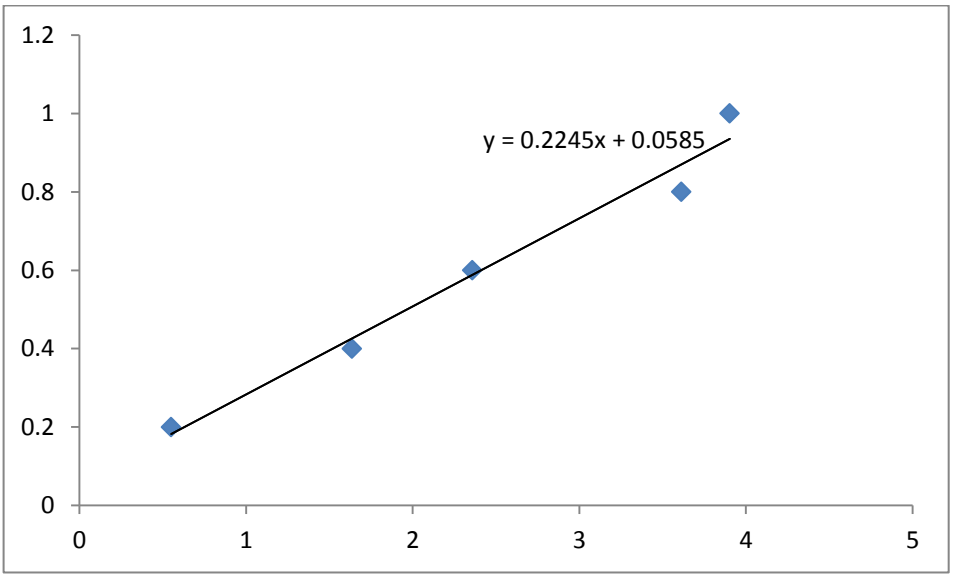


Figure 4.7: Standard graph with concentration on X-axis and OD values on Y-axis

4.3.4 Production of volatile compounds

Volatile compounds represent another element of the plant growth-promoting or pathogen inhibiting strategies of the PGPB. The volatiles are extremely small in size and can be easily diffused in the soil because of its porous structure and is hence to be extremely effective for rhizobacteria (Vespermann et al, 2012). The volatiles generated by *Streptomyces alboflavus* were previously reported to inhibit numerous fungi *in vitro* including, *Fusarium moniliforme, Aspergillus flavus* and *Pencillin citrinum* (Changlu Wang et al., 2013). In the present investigation, the growth of *Fusarium pseudograminearum* was inhibited by volatile compounds produced by strains NM-12 and NM-33. The effect of volatile components on the growth of *F. pseudograminearum* resulted in significant inhibition. Strains, NM-12 and NM-33, showed maximum inhibition of 32.40% and 26.24%, respectively (Fig. 4.7).

4.3.5 ACC deaminase and HCN producing genes

The above results suggest that NM-12 exhibits greater suppression of *F. pseudograminearum* when compared with NM-33. Strain NM-12 was therefore selected and further characterised to assess if it possesses genes encoding for the production of ACC deaminase and hydrogen cyanide (HCN). As seen in Figure 4.8, strain NM-12 showed the presence of genes encoding for the production of both these compounds (Fig. 4.8). Ethylene is a plant hormone mainly associated with senescence, cell death and ageing (Koyama, 2014). ACC is an immediate precursor for ethylene in plants and therefore ACC deaminase can breakdown ACC and limit the production of ethylene; ACC is therefore considered to be plant growth promoting. In fact, certain PGPB strains with showing ACC-deaminase enzyme activity were shown to be capable of breaking down ACC into α-ketobutyrate and ammonia which results in decreased levels of ACC (Tahir et al., 2006; Arshad et al., 2007). In general, reduced amount of ACC results in lowered ethylene production in plants.

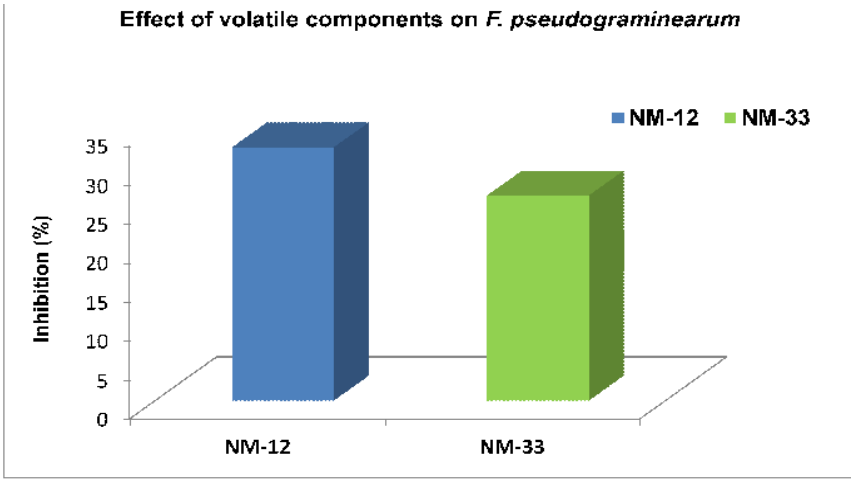


Figure 4.8: Percentage inhibition of Fusarium pseudograminearum growth on PDA plates by volatile compounds from bacterial isolates NM-12 and NM-33

Cyanogen activity (HCN production) by PGPB has been suggested to play a significant role in pathogen control and antibiosis (Voisard et al. 1989; Devi et al. 2007). Further, HCN production by some bacteria has been shown to increase with supplementation of soil with glycine (Owen and Zdor, 2001). In one study HCN deficient mutants of *Pseudomonas fluorescens* strain CHAO were shown to have a decreased ability to control tobacco black rot (Stuz et al., 1986). Some researchers suggest that PGPB which can synthesize antibiotics as well as HCN are more suitable for biocontrol use as having multiple antagonistic traits makes it more difficult for pathogens to acquire resistance (Gilchik, 2012). Therefore, ACC deaminase and HCN production ability of strain NM-12 further corroborates its suitability as a PGPB to control crown rot of chickpea and wheat.

4.4 Conclusions

From the results obtained in the current study, the effect of secondary metabolites and volatiles on the growth of *F. pseudograminearum* was significant, therefore the characterization of those metabolites and volatile compounds is critical for the assessment of the potential applicability of strains NM-12 and NM-33 as biocontrol agents. Strain NM-12 was able to produce IAA at different concentrations even up to 6% salinity (no IAA production was observed in the case of strain NM-33). Further, siderophore production was moderate under control conditions but increased at high salt concerntration (6%). In contrast β- glucanase production was observed under normal as well as high salt concentrations. Interestingly, NM-12 which exhibited a greater ability to suppress the fungal pathogen was found to

possess genes encoding for both HCN and ACC, both of which are indirectly responsible for the plant growth promotion.

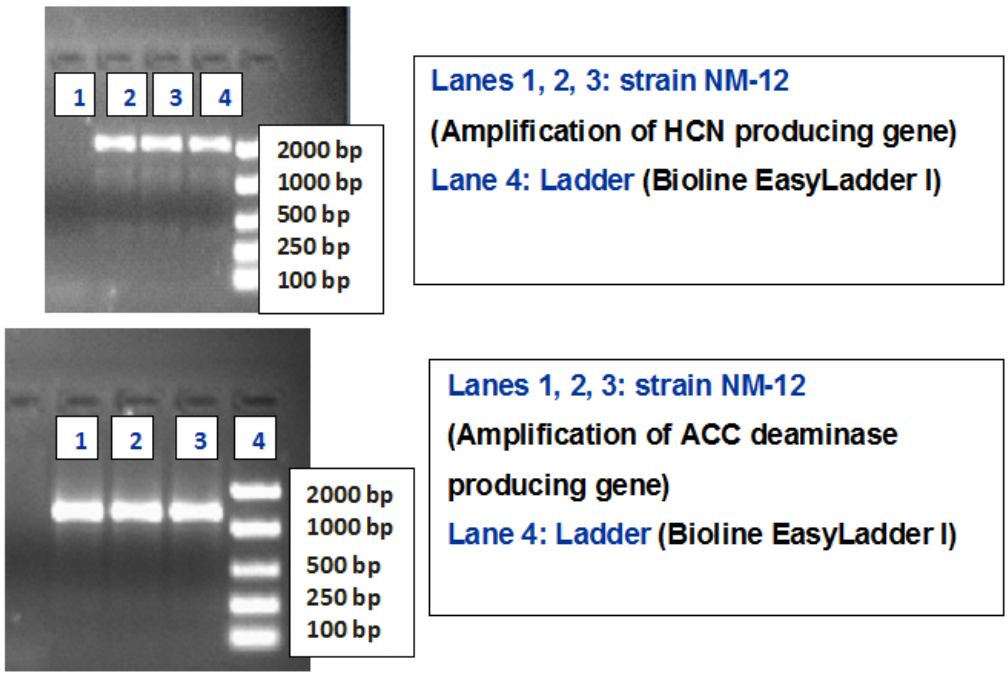


Figure 4.9: PCR gel image showing amplification of both HCN and ACC deaminase producing gene s in NM-12

Thus, the results from this study indicate that strains NM-12 and NM-33 represent good biocontrol candidates as both inhibit the growth of F. *pseudograminearum*, the causal agent of crown rot in chickpea and wheat. Since salinity generally has a negative impact on secondary metabolite production in PGPB and is considered as a crucial factor; the fact that these strains were still capable of producing antifungal activity even at high salinity suggests that these two strains (NM-12 and NM-33) could be used as potential biocontrol agents even in saline soils. However, these two isolates require further *in vivo* screening to fully assess their potential as biocontrol agents. Nonetheless, considering the fact that PGPB are eco-friendly, isolation of new effective PGPB represents a better alternative to the

continuing use of chemical fertilizers/pesticides to promote sustainable agriculture systems in crop production/protection.

5 CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

Fusarium Crown Rot (FCR) is a major disease that impacts crop productivity in Australia resulting in significant financial losses of up to $80 million AUD. Further, mycotoxins might be produced by FCR infecting plants and these in turn pose severe safety threat for humans. Biocontrol is an effective strategy that can be used to reduce FCR infections, suppressing its causative pathogen. Biocontrol might also be considered a superior alternative compared to the use of pesticides as it is environmentally friendly.

In this context, this study aimed at screening bacteria from Australian soils and identifying Plant Growth Promoting Bacteria (or PGPB) isolates that can suppress *F. pseudograminearum*, the causal agent of crown rot. Only wheat and chickpea plants were considered for the purpose of this study as both of these are major winter pulse crops grown in Australia. Australia is also the largest exporter of wheat and chickpea and this made both these crops ideal for study.

The study was conducted with the help of a series of assays; soil samples were collected from rhizosphere of three soils, each from different regions of Victoria, Australia (Perry Bridge, Lardner and Rosedale). The results of the isolation assay revealed the maximum diversity of the isolated bacteria was on tryptone soy agar. Nutrient agar also facilitated maximum recovery of bacteria from soil. Interestingly, most of the effective bacterial isolates which produced zones of clearance on plates inoculated with fungus and bacteria were from the same location, Perry Bridge, Victoria.

After the bacterial isolates were obtained, sibling strains were eliminated and a total of 32 strains were selected for the dual cultural assay. The results of this assay revealed that the bacteria varied in terms of their abilities to suppress the radial growth rate of the fungus (*F. pseudograminearum*) as compared to the control. Strain NM-12 demonstrated the best results in terms of reducing the radial growth rate of the fungus (1.6 cm) as compared to the control (8.4 cm). Strain NM-33 was the second most promising strain as it restricted the radial growth of the pathogen to 2.6 cm.

Next, a culture filtrate assay was used to determine the extent to which selected isolates might suppress the fungal pathogen. Bacterial strains that showed aggressive suppression on the fungus during the dual culture assay were processed further, and assessed in terms of a cell culture filtrate assay. The results obtained from this assay showed that both effective bacterial strains NM-12 (*Bacillus* spp.) and NM-33 (*Stenotrophomonas rhizophila)* suppressed the fungal pathogen with 79% and 74% efficiency, respectively. Commenting on the growth phase of the bacteria that resulted in greatest inhibition of fungal growth, culture filtrate from both bacterial strains were most effective in terms of inhibition on the third day of incubation, when in other words, stationary growth phase had been reached. Based on the results obtained, strain NM-12 (*Bacillus subtilis*) and NM-33 (*Stenotrophomonas rhizophila*) exhibited significant broad spectrum biocontrol activity. Grosu et al. (2015) found that a *Bacillus* spp. strain inhibited *F. graminearum* schwabe (which also causes crown rot) and *F. culmorum*. This strain was more potent compared to a *Bacillus amyloliquefaciens* strain. This was

attributed to the ability of *Bacillus spp*. to produce glucanase, chitinase and ilutirin antifungal compounds.

The impact of the growth of these two isolates on the survival of fungal spores in broth culture was determined by assessing the number of fungal spores using a haemocytometer. The results of the experiment clearly indicated that a combination of both strains (NM-12 & NM-33) was superior in reducing the fungal spore count as compared to results obtained by either of the strains alone. None of the previous studies reported this type of dual culture broth assay to determine the inhibition of fungal growth by monitoring the spore count of the fungal pathogen in the presence of bacteria. In our knowledge, this is the first attempt to understand the inhibition effect of bacteria on fungal growth in liquid culture by inoculating equal concentration and amount of bacterial and fungal cells.

The idea of co-culturing microorganisms to produce novel antifungal compounds has previously been shown to be successful. Researchers reported the production of a new antibiotic, pestalone, first isolated from a mixed fermentation culture of a marine deuteromycete, (*Pestalotia* sp.) with a marine bacterium. This antibiotic was not detected when either of the strains was cultured separately.

Further assays were conducted in order to try and identify the basis for the antifungal activity. The concentrations of antifungal/growth-promoting compounds namely, β-glucanase, indole acetic acid (IAA) (a plant hormone), siderophores, and volatile components present in the culture filtrates of the two isolates exhibiting antifungal activity were assessed.

Further, to determine if these four antifungal/growth-promoting compounds could be produced under salt stressed conditions, a comparison of their activity during growth of the two isolates under increasing salinity was undertaken. The results revealed that both strains, NM-12 and NM-33 produced highest amounts of siderophores at 6% salinity. In addition, it was observed that the production of IAA was zero at 8% and 10% salinity. NM-33 did not show any IAA activity under any conditions. The maximum production of IAA was seen in NM-12 at normal saline conditions; the production of IAA decreased with increasing salt concentration. The production of IAA by NM-12 was observed up to 6% salinity.

In contrast, the production of siderophores was greatest at high salt concentrations when compared to normal conditions. The highest amount of siderophore production was produced by NM-12 and NM-33 at 6% salinity. Isolates NM-12 and NM-33 were also tested for the production of β-glucanase under normal and saline conditions. For strain NM-12, the production of β-glucanase was observed to be 252.3 units of activity at normal and 10% salinity with maximum value at 4% (302.7 units). NM-33 showed maximum enzyme activity in normal saline conditions when compared to increased salt concentrations. Finally, the impact of volatile compounds on fungal suppression was studied. The effect of volatile components on inhibiting the growth of *F. pseudograminearum* was significant. NM-12 & NM-33 showed maximum inhibition of 32.40% and 26.24%, respectively. The PGP attributes such as indole acetic acid (IAA), siderophore and β-glucanase production can be said to be possible causes of enhanced morphological observations by these two isolates. The overall growth of the plant and elongation of root are said to get enhanced due to the

IAA – producing microorganisms (Patten and Glick 2002), but the main mechanism of acting of siderophore producers is by binding Fe^{3+} from the environment and making it available to the plant (Wang et al. 1993). In the present investigation, such enhanced activities were found in these two isolates under normal and saline conditions. Looking at results obtained with the help of a series of assays, it is evident that salinity helps improve the fungal suppression capability of metabolites produced by PGPB (especially isolate NM-12).

These strains can thus be used as effective biocontrol agents in saline soils. The two isolates that were incorporated in the research were found to be well adapted to the overall rhizosphere environment and their main role was found to be promotion of growth of plants. Hence, the possibility of using these isolates as PGP agents while also being used as biocontrol agents for controlling crown rot, is possible. Production of cell wall degrading enzymes, antibiosis and also plant growth promoting hormones are some of the key mechanisms observed due to PGP and also the antifungal activities demonstrated by these two isolates. Broad spectrum PGP and biocontrol agents (and their secondary metabolites) hence provide new as well as effective strategies that can be used for controlling different types of pathogens as well as insect pests. Broad spectrum antifungal activity has been demonstrated by a few of the overall broad spectrum agents, mostly belonging to *Bacillus* and *Stenotrophomonas* spp. (Hass and Keel 2003; Viji et al. 2003). Secondary metabolites of *P. aeruginosa* possess antifungal, PGP and biocontrol activities (Bano and Musarrat 2003). ICRISAT has identified actinomycetes (isolated from various herbal composts) and bacteria that inhibit *Fusarium oxysporum* f. sp. *ciceri*, *Rhizoctonia bataticola*, *M.*

phaseolina, *Helicoverpa armigera* and *Spodoptera litura* (Gopalakrishnan et al. 2011b,c,d). The two potential isolates can be hence said to be effective and beneficial for possible discovery of different novel secondary metabolites that can be important for PGP as well as biocontrol applications.

5.1 FUTURE PERSPECTIVES

Current application of the chemical pesticides that are harmful to the environment, is limited to controlling the stress factors and also improving the overall production of crop and hence future production of crop requires strategies that are environment friendly. The tool of biocontrol has been found to exhibit high and broad range of stress control and has also been found to be effective for improving overall production of crop by ensuring that no adverse impact on the environment is caused due to the chemical pesticides. The main goals of this study were to characterize some of the effects of microbial interaction on plants and elucidate some of the mechanisms. Effectiveness of these strains however also needs to be established with the help of additional studies in this domain. Therefore, this study helped in opening new research avenues in context of using effective biocontrol strategies along with contributing to the existing body of evidence on the topic. Studies to date in this project have provided several insights regarding the concept of sustainable production with emphasis on *Bacillus* and *Stenotrophomonas* spp. promoting plant growth and antagonising pathogens.

Future research can be thus focused on the study and use of rhizosphere biology such that new settings can be developed for reliable development of molecular, as well as biotechnological approaches that can help in enhancing the overall

knowledge of crucial molecules that are operational at the time of interaction taking place among plant microbes, which usually result into an advantageous interaction. In addition, even the ability to understand and comprehend microbial signals for eliciting high resistance to pathogen in plants via the method of Induced Systemic Resistance remains a major issue. Hence, it can be stated that a better understanding of the overall rhizosphere biology as well biodiversity with respect to the application of PGPR and BCA at scale is essential. An effective strategy that can be beneficial for minimising the adverse impacts caused by stress on growth of plants can be the use of multi-strain bacterial inoculation (Consortium), however the practice also require establishment of multiple prerequisites for effective combinations. The studies that are currently present with respect to the study of bacterial volatile compounds (known and unknown compounds) can be helpful in addressing the mode of action as observed for multiple compounds and also for identification of effective combinations. Use of volatile compounds can be facilitated as the antibiotics or inducers when induced against pathogens in the field of agriculture. Effectiveness of these strains however also needs to be established with the help of additional studies in this domain. Therefore, this study helped in opening new research avenues in context of use of effective biocontrol strategies along with contributing to the existing body of evidence on the topic.

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